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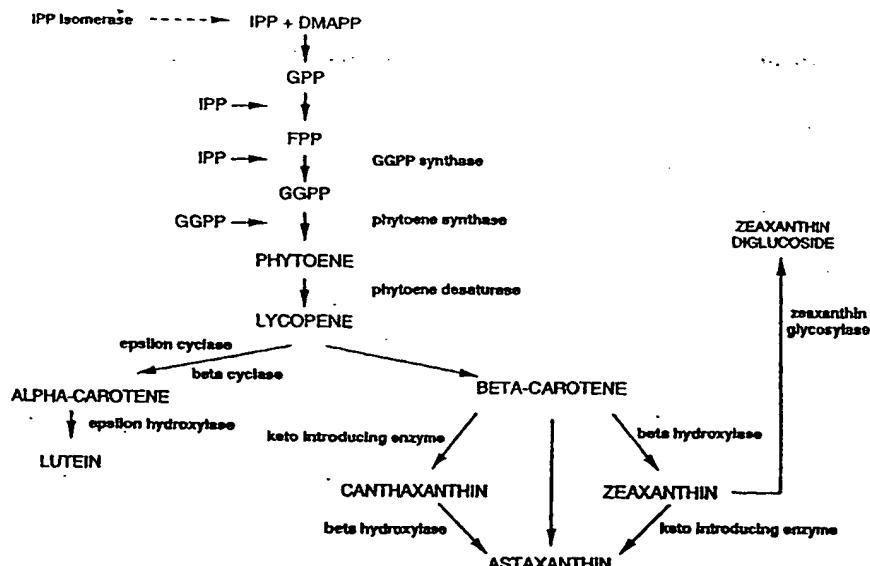
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## (54) Title: METHODS FOR PRODUCING CAROTENOID COMPOUNDS, AND SPECIALITY OILS IN PLANT SEEDS



## (57) Abstract

Methods are provided for producing plants and seeds having altered carotenoid compositions by transforming host plants with constructs having a transcriptional initiation region from a gene expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from at least one carotenoid biosynthesis gene coding region, and a transcriptional termination region. The methods find particular use in increasing the carotenoid content in oilseed plants.

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## METHODS FOR PRODUCING CAROTENOID COMPOUNDS, AND SPECIALITY OILS IN PLANT SEEDS

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This application is a continuation-in-part of Application Serial No.08/908,758 filed August 8, 1997 which is a continuation-in-part of Application Serial No.60/024,145 filed August 9, 1996.

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### FIELD OF THE INVENTION

The invention relates to genetic modification of plants, plant cells and seeds, particularly altering carotenoid biosynthesis, and fatty acid composition.

### BACKGROUND OF THE INVENTION

15

Carotenoids are pigments with a variety of applications. They are yellow-orange-red lipids which are present in green plants, some molds, yeast and bacteria. Carotenoid hydrocarbons are referred to as carotenes, whereas oxygenated derivatives are referred to as xanthophylls. The carotenoids are part of the larger isoprenoid biosynthesis pathway which, in addition to carotenoids, produces such compounds as chlorophyll and tocopherols, Vitamin E active agents. The carotenoid pathway in plants produces carotenes, such as  $\alpha$ - and  $\beta$ -carotene, and lycopene, and xanthophylls, such as lutein.

20

The biosynthesis of carotenoids involves the condensation of two molecules of the C<sub>20</sub> precursor geranyl PP, to yield the first C<sub>40</sub> hydrocarbon phytoene. In a series of sequential desaturations, phytoene yields lycopene. Lycopene is the precursor of the cyclic carotenes,  $\beta$ -carotene and  $\alpha$ -carotene. The xanthophylls, zeaxanthin and lutein are formed by hydroxylation of  $\beta$ -carotene and  $\alpha$ -carotene, respectively.

β-carotene, a carotene whose color is in the spectrum ranging from yellow to orange, is present in a large amount in the roots of carrots and in green leaves of plants. β-carotene is useful as a coloring material and also as a precursor of vitamin A in mammals. Current methods for commercial production of β-carotene include 5 isolation from carrots, chemical synthesis, and microbial production.

A number of crop plants and a single oilseed crop are known to have substantial levels of carotenoids, and consumption of such natural sources of carotenoids have been indicated as providing various beneficial health effects. The below table provides levels of carotenoids that have been reported for various plant 10 species.

**CAROTENOID CONTENTS OF VARIOUS CROPS**  
( $\mu\text{g/g}$ )

15	<u>Crop</u>	<u>Beta-Carotene</u>	<u>Alpha-Carotene</u>	<u>Lycopene</u>	<u>Lutein</u>	<u>Total</u>
	Carrots	30-110	10-40	0-0.5	0-2	65-120
	Pepper (gr)	2	-	-	2	8
	Pepper (red)	15	1	-	-	200
	Pumpkin	16	0.3	tr	26	100
20	Tomato	3-6	-	85	-	98
	Watermelon	1	tr	19	-	25
	Marigold petals	5	4	-	1350	1500
	Red palm oil	256	201	8	-	545

25 The pathway for biosynthesis of the carotenoids has been studied in a variety of organisms and the biosynthetic pathway has been elucidated in organisms ranging from bacteria to higher plants. See, for example, Britton, G. (1988) *Biosynthesis of carotenoids*, p. 133-182, In T.W. Goodwin (ed.), Plant pigments, 1988. Academic Press, Inc. (London), Ltd., London. Carotenoid biosynthesis genes have also been 30 cloned from a variety of organisms including *Erwinia uredovora* (Misawa *et al.* (1990) *J. Bacteriol.* 172:6704-6712; *Erwinia herbicola* (Application WO 91/13078,

Armstrong *et al.* (1990) *Proc. Natl. Acad. Sci., USA* 87:9975-9979); *R. capsulatus* (Armstrong *et al.* (1989) *Mol. Gen. Genet.* 216:254-268, Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196:1414-1421); *Thermus thermophilus* (Hoshino *et al.* (1993) *Appl. Environ. Microbiol.* 59:3150-3153); the cyanobacterium 5 *Synechococcus* sp. (Genbank accession number X63873). See also, application WO 96/13149 and the references cited therein.

While the genes have been elucidated, little is known about the use of the 10 genes in plants. Investigations have shown that over expression or inhibition of expression of the plant phytoene synthase (Psy1) gene in transgenic plants can alter carotenoid levels in fruits. See, Bird *et al.* (1991) *Biotechnology* 9:635-639; Bramley *et al.* (1992) *Plant J.* 2:343-349; and Fray and Grierson (1993) *Plant Mol. Biol.* 22:589-602. Further, as reported by Fray *et al.* (1995) *The Plant Journal* 8:693-701, constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway.

15 Application WO 96/13149 reports on enhancing carotenoid accumulation in storage organs such as tubers and roots of genetically engineered plants. The application is directed towards enhancing colored native carotenoid production in specific, predetermined non-photosynthetic storage organs. The examples of the application are drawn to increasing colored carotenoids in transformed carrot roots 20 and in orange flesh potato tubers. Both of these tissues are vegetative tissues, not seeds, and natively have a high level of carotenoids.

Carotenoids are useful in a variety of applications. Generally, carotenoids are useful as supplements, particularly vitamin supplements, as vegetable oil based food products and food ingredients, as feed additives in animal feeds and as colorants. 25 Specifically, phytoene finds use in treating skin disorders. See, for example, U.S. Patent No. 4,642,318. Lycopene,  $\alpha$ - and  $\beta$ -carotene are used as food coloring agents. Consumption of  $\beta$ -carotene and lycopene has also been implicated as having

preventative effects against certain kinds of cancers. In addition, lutein consumption has been associated with prevention of macular degeneration of the eye.

Plant oils are useful in a variety of industrial and edible applications. Novel vegetable oils compositions and/or improved means to obtain oils compositions, from biosynthetic or natural plant sources are needed. Depending upon the intended oil use, various different fatty acid compositions are desired. The demand for modified oils with specific fatty acid compositions is great, particularly for oils high in oleic acid. See, Haumann, B. F. (1996) *INFORM* 7:320-334. As reported by Haumann, the ideal frying oil would be a low-saturate, high oleic and low linolenic oil.

10 Furthermore, studies in recent years have established the value of monounsaturated fatty acids as a dietary constituent.

Attempts have been made over the years to improve the fatty acid profiles of particular oils. For example, the oxidative stability of vegetable oil is related to the number of double bonds in its fatty acids. That is, molecules with several double bonds are recognized to be more unstable. Thus, scientists have attempted to reduce the content of  $\alpha$ -linolenic acid in order to improve shelf life and oxidative stability, particularly under heat.

It is apparent that there is needed a method for producing significant levels of carotenoid compounds in crop plants and particularly in plant seeds. It would additionally be beneficial to alter the fatty acid content of the plants and seeds. Such altered seed products would be useful nutritionally as well as provide a source for producing more stable oils. There is no report of methods to substantially altering the levels and composition of carotenoids produced in a plant seed, particularly with respect to increasing the level of production of carotenoids. There is therefore needed, a useful method for altering carotenoid levels in plants, particularly seeds, and for producing oils with modified carotenoid composition and/or content.

#### SUMMARY OF THE INVENTION

Transformed plants, plant cells and seeds having altered carotenoid levels and/or modified fatty acid compositions are provided. The plants, plant cells and seeds are transformed with at least one carotenoid biosynthesis gene, or a combination thereof. Methods for making and using the transformed compositions of the invention are also provided. Methods find use in altering carotenoid levels in plants, particularly seeds, as well as increasing particular compounds for molecular farming, such as for production of particular carotenoids. At the same time, the transformed compositions, particularly seeds, provide a source of modified oils, which oils may be extracted from the seeds in order to provide an oil product comprising a natural source of various carotenoids, carotenoid mixtures. In a particular aspect of the present invention, transformed seed can provide a source for particular carotenoid compounds and/or for modified speciality oils having altered carotenoid compositions and/or altered fatty acid composition, particularly having increased levels of oleic acid and decreased levels of linoleic and linolenic acids.

15

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence of the SSU/*crtB* fusion sequence.

Figure 2 presents constructs for expression of carotenoid biosynthesis genes in plant seeds. Figure 2A shows plasmid pCGN3390 which contains the napin promoter operably linked to the SSU/*crtB* sequence. Figure 2B shows plasmid pCGN3392 which contains the napin promoter operably linked to the SSU/*crtE* sequence. Figure 2C shows plasmid pCGN9010 which contains the napin promoter operably linked to the SSU/*crtI* sequence. Figure 2D shows plasmid pCGN9009 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to the SSU/*crtI* sequence. Figure 2E shows plasmid pCGN9002 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to an antisense epsilon cyclase sequence. Figure 2F shows plasmid pCGN9017 which contains the napin promoter

operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to an antisense beta cyclase sequence. Figure 2G shows plasmid pCGN6204 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to the SSU/*crtW* sequence. Figure 2H shows plasmid 5 pCGN6205 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to the *crtZ* sequence. Figure 2I shows plasmid pCGN6206 which contains the napin promoter operably linked to the SSU/*crtB* sequence, the napin promoter operably linked to the *crtW* sequence and the napin promoter operably linked to the *crtZ* sequence.

10 Figure 3 shows the results of analyses of saponified samples for control seeds.

Figure 4 shows the results of analyses of saponified samples for pCGN3390 transformed seeds.

15 Figure 5 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates that the increase in 18:1 fatty acids correlates with a decrease in 18:2 and 18:3.

Figure 6 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates that the increase in 18:1 correlates with an increase in both 18:0 and 20:0, but little effect is seen in 16:0.

20 Figure 7 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates the increase in 18:0 correlates well with an increase in 20:0.

Figure 8 shows a carotenoid biosynthesis pathway.

Figure 9 provides sequence of *B. napus* epsilon cyclase cDNA clone 9-4.

Figure 10 provides sequence of *B. napus* epsilon cyclase cDNA clone 7-6.

Figure 11 provides sequence of a *B. napus* beta cyclase cDNA clone.

25 Figure 12 provides T2 seed analysis of 3390 transformed *Brassica napus* plants.

Figure 13 provides T3 seed analysis of 3390 transformed *Brassica napus* plants.

Figure 14 provides T2 seed analysis of 9002 transformed *Brassica napus* plants.

Figure 15 shows the nucleotide sequence of the SSU/crtZ fusion sequence.

Figure 16 shows the nucleotide sequence of the SSU/crtW fusion sequence.

5 Figure 17 shows the HPLC trace for detection of xanthophylls from extractions from seed of 6204 transgenic lines.

#### DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, methods for increasing production of 10 carotenoid compounds, as well as for altering fatty acid compositions in a plant, particularly in plant seeds, are provided. The method involves transforming a plant cell with at least one carotenoid biosynthesis biosynthesis gene. This has the effect of altering carotenoid biosynthesis, particularly increasing the production of downstream products, as well as providing novel seed oils having desirable fatty acid 15 compositions. A second gene can then be utilized to shunt the metabolic activity to the production of particular carotenoid, or to further alter the fatty acid composition.

Surprisingly, it has been found that transformation of a plant with an early 20 carotenoid biosynthesis gene leads to a significant increase in the flux through the carotenoid pathway resulting in an increase in particular carotenoids. That is, there is an increase in the metabolic activity that can be further manipulated for the production of specific carotenoids. In addition, the transformed seeds may demonstrate altered fatty acid compositions as the result of the carotenoid gene expression, such as seen with the seeds described herein from plants transformed with a phytoene synthase gene.

25 Thus, using the methods of the invention, seeds are provided which produce high levels of particular carotenoids and/or produce speciality oils having a desired fatty acid composition. In oilseed *Brassica*, for example, transformation with an early carotenoid biosynthesis gene leads to seeds having significant increases in the levels

of  $\alpha$ -carotene,  $\beta$ -carotene and lutein. In addition, the *Brassica* seeds demonstrate an altered fatty acid composition and yield a vegetable oil which has increased oleic acid content and decreased linoleic and linolenic acid content. Thus, the transformed seed can provide a source of carotenoid products as well as modified seed oil. In this 5 manner, modified speciality oils can be produced and new sources of carotenoids for extraction and purification are provided.

The oils of the present invention also provide a substantial improvement with respect to stability as compared to two other major plant sources of carotenoids, marigold petals and red palm oil (mesocarp). Although instability is observed in 10 seeds stored in air at room temperature as demonstrated by loss of approximately 20-30% of total carotenoids after 4 weeks of storage, the loss after 1-2 weeks is only 15 10%. Palm mesocarp, by contrast, must be processed within a day or two of harvest in order to avoid major losses of carotenoids. Furthermore, the carotenoid decomposition in the seeds of the present invention may be reduced significantly by storage of the seeds under nitrogen.

For the production of a seed having an increase in carotenoid biosynthesis, transformation of the plant with an early carotenoid biosynthesis gene is sufficient. By early carotenoid biosynthesis gene is intended geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate (IPP) 20 isomerase. A variety of sources are available for the early carotenoid biosynthesis genes and for the most part, a gene from any source can be utilized. However, it is recognized that because of co-suppression, the use of a plant gene native to the target host plant may not be desirable where increased expression of a particular enzyme is desired.

25 A number of early carotenoid biosynthesis genes, also referred herein as DNA sequences derived from carotenoid biosynthesis gene coding regions, have been isolated and are available for use in the methods of the present invention. See, for example:

IPP isomerase has been isolated from: *R. Capsulatus* (Hahn *et al.* (1996) *J. Bacteriol.* 178:619-624 and the references cited therein), GenBank Accession Nos. U48963 and X82627, *Clarkia xantiana* GenBank Accession No. U48962, *Arabidopsis thaliana* GenBank Accession No. U48961, *Schizosaccharomyces pombe* GenBank Accession No. U21154, human GenBank Accession No. X17025, *Kluyveromyces lactis* GenBank Accession No. X14230;

5 geranylgeranyl pyrophosphate synthase from *E. Uredovora* Misawa *et al.* (1990) *J. Bacteriol.* 172:6704-6712 and Application WO 91/13078; and from plant sources, including white lupin (Aitken *et al.* (1995) *Plant Phys.* 108:837-838), bell

10 pepper (Badillo *et al.* (1995) *Plant Mol. Biol.* 27:425-428) and *Arabidopsis* (Scolnik and Bartley (1994) *Plant Physiol.* 104:1469-1470; Zhu *et al.* (1997) *Plant Cell Physiol.* 38:357-361).

phytoene synthase from a number of sources including *E. Uredovora*, *Rhodobacter capsulatus*, and plants Misawa *et al.* (1990) *J. Bacteriol.* 172:6704-6712, 15 GenBank Accession No. D90087, Application WO 91/13078, Armstrong *et al.* (1989) *Mol. Gen. Genet.* 216:254-268, Armstrong, G. A. "Genetic Analysis and regulation of carotenoid biosynthesis. In R. C. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), *Anoxygenic photosynthetic bacteria; advances in photosynthesis*. Kluwer Academic Publishers, Dordrecht, The Netherlands, Armstrong *et al.* (1990) *Proc. Natl. Acad. Sci USA* 87:9975-9979, Armstrong *et al.* (1993) *Methods Enzymol.* 214:297-311, 20 Bartley and Scolnik (1993) *J. Biol. Chem.* 268:27518-27521, Bartley *et al.* (1992) *J. Biol. Chem.* 267:5036-5039, Bramley *et al.* (1992) *Plant J.* 2:291-343, Ray *et al.* (1992) *Plant Mol. Biol.* 19:401-404, Ray *et al.* (1987) *Nucleic Acids Res.* 15:10587, 25 Romer *et al.* (1994) *Biochem. Biophys. Res. Commun.* 196:1414-1421, Karvouni *et al.* (1995) *Plant Molecular Biology* 27:1153-1162, GenBank Accession Nos. U32636, Z37543, L37405, X95596, D58420, U32636, Z37543, X78814, X82458, S71770, L27652, L23424, X68017, L25812, M87280, M38424, X69172, X63873, and

X60441, Armstrong, G. A. (1994) *J. Bacteriol.* 176:4795-4802 and the references cited therein; and,

5 phytoene desaturase from bacterial sources including *E. uredovora* Misawa *et al.* (1990) *J. Bacteriol.* 172:6704-6712, and Application WO 91/13078 (GenBank Accession Nos. L37405, X95596, D58420, X82458, S71770, and M87280); and from plant sources, including maize (Li *et al.* (1996) *Plant Mol. Biol.* 30:269-279), tomato (Pecker *et al.* (1992) *Proc. Nat. Acad. Sci.* 89:4962-4966 and Araci *et al.* (1994) *Plant Physiol.* 106:789), and *Capsicum annuum* (bell peppers) (Hugueney *et al.* (1992) *J. Biochem.* 209: 399-407), GenBank Accession Nos. U37285, X59948, X78271, and 10 X68058).

See, generally, Misawa *et al.* (1990) *J. of Bacteriology* 172:6704-6712, E.P. 0393690 B1, U.S. Patent No. 5,429,939, Bartley *et al.* (1992) *J. Biol. Chem.* 267:5036-5039, Bird *et al.* (1991) *Biotechnology* 9:635-639, and US Patent No. 5,304,478, which disclosures are herein incorporated by reference.

15 Transformation with an early carotenoid gene, (herein referred to as the primary gene), increases the biosynthetic activity of the carotenoid pathway, and can lead to increased production of particular carotenoids such as for example,  $\alpha$ - and  $\beta$ -carotene. As described in more detail in the following examples, by expression of phytoene synthase as the primary gene, large increases in the carotenoid content 20 generally; and particularly in the levels of  $\alpha$ - and  $\beta$ -carotene, are obtained in seeds of transformed plants. Oil comprising the carotenoids so produced may be extracted from the seeds to provide a valuable source of  $\alpha$ - and  $\beta$ -carotenes. Such an oil may find use as a food colorant, for example to add color to margarines, or as a food oil. An edible food oil with high  $\alpha$ - and  $\beta$ -carotene levels is of interest for prevention of 25 Vitamin A deficiency which can result in night blindness. Thus, production of transformed plants and extraction of the high  $\alpha$ - and  $\beta$ -carotene oil to provide a useful food oil is particularly desirable in regions where night blindness is a widespread problem, such as in India and Asia.

In addition to high  $\alpha$ - and  $\beta$ -carotene levels, levels of other carotenoids are also increased in the oils exemplified herein. For example, lutein levels are increased in seeds from plants transformed with a phytoene synthase gene, as well as in seeds from plants transformed with a GGPP synthase gene, *crtE* (3392), or with phytoene desaturase, *crtI* (9010).

Furthermore, additional primary genes may be expressed to provide for even greater flux through the carotenoid pathway. For example, in oilseed *Brassica* seeds transformed with a phytoene synthase gene as described herein, increased levels of phytoene are observed. Thus, increasing the expression of phytoene desaturase as well as phytoene synthase may result in further increases in the levels of carotenoids, such as  $\alpha$ - and  $\beta$ -carotene and lutein, produced. Such further modification of carotenoid composition is demonstrated here in transgenic plant seeds transformed with pCGN9009 for the expression of *crtB* and *crtI* genes. Additionally, plants expressing both phytoene synthase and GGPP synthase genes are desirable. Such plants may demonstrate even greater flux through the carotenoid pathway as indicated by the increased production of chlorophyll observed in plants of the present invention which have been transformed to express a GGPP synthase gene (*crtE*) in the absence of *crtB* overexpression.

Interestingly, plants expressing a GGPP synthase gene did not have significant modifications of the tocopherol content. Since GGPP is a branch point of the carotenoid, chlorophyll and tocopherol pathways in plants, these observations suggest that the next enzymatic step in tocopherol biosynthesis, catalyzed by GGPP hydrogenase, is a rate limiting step for tocopherol production. Thus, providing for increased expression of GGPP hydrogenase, alone or in conjunction with increased expression of GGPP synthase would be expected to result in an increase of flux to the tocopherol pathway.

Also of interest are plants which are transformed to express three early carotenoid biosynthesis genes, *crtB*, *crtE*, and *crtI*. Plants expressing two or three

different carotenoid biosynthesis genes may be produced by either transforming a plant with a construct providing for expression of the desired genes, using a multiple gene construct or by cotransformation with multiple constructs, or by crossing plants which contain the different desired genes.

5        In addition to the production of the carotenoids described herein, once the biosynthetic activity has been increased by expression of the primary carotenoid biosynthesis gene or genes, the pathway can be diverted for the production of specific compounds. The diversion involves the action of at least one second gene of interest, (the secondary gene). The secondary gene can encode an enzyme to force the

10      production of a particular compound or alternatively can encode a gene to stop the pathway for the accumulation of a particular compound. For forcing the production of a particular compound, expression of a carotenoid biosynthesis gene in the pathway for the desired carotenoid compound is used. Genes native or foreign to the target plant host may find use in such methods, including, for example, carotenoid

15      biosynthesis genes from sources other than higher plant, such as bacteria, including *Erwinia* and *Rhodobacter* species. For stopping the pathway in order to accumulate a particular carotenoid compound, the secondary gene will provide for inhibition of transcription of a gene native to the target host plant, wherein the enzyme encoded by the inhibited gene is capable of modifying the desired carotenoid compound.

20      Inhibition may be achieved by transcription of the native gene to be inhibited in either the sense (cosuppression) or antisense orientation of the gene.

For example, for alteration of the carotenoid composition towards the accumulation of higher levels of  $\beta$ -carotene derived carotenoids, such as zeaxanthin, zeaxanthin diglucoside, canthaxanthin, and astaxanthin, inhibition of lycopene epsilon cyclase is desired to prevent accumulation of alpha carotene and its derivative carotenoids, such as lutein. In addition, overexpression of lycopene  $\beta$ -cyclase may be used to increase the accumulation of  $\beta$ -carotene derived carotenoids. Thus, antisense lycopene epsilon cyclase and lycopene  $\beta$ -cyclase are examples of sequences which

find use in secondary gene constructs of interest in the present invention.

Furthermore, in conjunction with the inhibition of lycopene epsilon cyclase, increased expression of additional secondary genes may be desired for increased accumulation of a particular beta-carotene derived carotenoid. For example, increased  $\beta$ -carotene hydroxylase expression is useful for production of zeaxanthin, whereas increased  $\beta$ -carotene hydroxylase and keto-introducing enzyme expression is useful for production of astaxanthin. Alternatively, for accumulation of lycopene, inhibition of lycopene beta cyclase or of lycopene epsilon cyclase and lycopene beta cyclase is desired to reduce conversion of lycopene to alpha- and beta-carotene.

Thus, the carotenoid pathway can be manipulated by expression of carotenoid biosynthesis genes to increase production of particular carotenoids, or by decreasing levels of a particular carotenoid by transformation with antisense DNA sequences which prevent the conversion of a selected precursor compound into the next carotenoid in the pathway.

Secondary genes of interest in the present application include but are not limited to:

$\beta$ -carotene hydroxylase or *crtZ* (Hundle *et al.* (1993) *FEBS Lett.* 315:329-334, GenBank Accession No. M87280) for the production of zeaxanthin; genes encoding keto-introducing enzymes, such as *crtW* (Misawa *et al.* (1995) *J. Bacteriol.* 177:6575-6584, WO 95/18220, WO 96/06172) or  $\beta$ -C-4-oxygenase (*crtO*; Harker and Hirschberg (1997) *FEBS Lett.* 404:129-134) for the production of canthaxanthin; *crtZ* and *crtW* or *crtO* for the production of astaxanthin;  $\epsilon$ -cyclase and  $\epsilon$ -hydroxylase for the production of lutein;  $\epsilon$ -hydroxylase and *crtZ* for the production of lutein and zeaxanthin; lycopene  $\beta$ -cyclase (*crtY*) (Hugueney *et al.* (1995) *Plant J.* 8:417-424, Cunningham FX Jr (1996) *Plant Cell* 8:1613-1626, Scolnik and Bartley (1995) *Plant Physiol.* 108:1343, GenBank Accession Nos. X86452,

L40176, X81787, U50739 and X74599) for increased production of  $\beta$ -carotene.

antisense lycopene  $\epsilon$ -cyclase (GenBank Accession No. U50738) for increased production of  $\beta$ -carotene;

antisense lycopene  $\epsilon$ -cyclase and lycopene  $\beta$ -cyclase for the production of 5 lycopene;

antisense plant phytoene desaturase for the production of phytoene; etc.

In this manner, the pathway can be modified for the high production of any particular carotenoid compound of interest, or for a particular subset of carotenoid compounds, such as xanthophylls. Such compounds include but are not limited to the 10 particular compounds described above, as well as,  $\alpha$ -cryptoxanthin,  $\beta$ -cryptoxanthin,  $\zeta$ -carotene, phytofluene, neurosporane, adonixanthin, echineneone, hydroxycanthaxanthin and the like. For a review of xanthophyll production, see Misawa, *et al.* (1995) *supra*). Using the methods of the invention, any compound of interest in the carotenoid pathway can be produced at high levels in a seed.

15 Secondary genes can also be selected to alter the fatty acid content of the plant for the production of speciality oils. For example, acyl-ACP thioesterase genes having specificity for particular fatty acid chain lengths may be used. See, for example, USPN 5,304,481, USPN 5,455,167, WO 95/13390, WO 94/10288, WO 92/20236, WO 91/16421, WO 97/12047 and WO 96/36719. Other fatty acid 20 biosynthesis genes of interest include, but are not limited to,  $\beta$ -keto acyl-ACP synthases (USPN 5,510,255), fatty acyl CoA synthases (USPN 5,455,947), fatty acyl reductases (USPN 5,370,996) and stearoyl-ACP desaturases (WO 91/13972).

Of particular interest is the use of a mangosteen acyl-ACP thioesterase as a 25 secondary gene for fatty acid content modification. As described in WO 96/36719 and WO 97/12047, a high stearate content may be obtained in seeds by expression of a mangosteen acyl-ACP thioesterase. To combine the high oleic acid trait of the 3390 plants described herein with the 5266 high stearate plants described in WO 97/12047, crosses were made between 3390-1 and 5266-35 and between 3390-1 and 5266-5.

Seeds resulting from these crosses contained oil having a high stearate, low linoleic, low linolenic and high carotenoid phenotype.

Any means for producing a plant comprising the primary gene or both the primary and secondary genes are encompassed by the present invention. For example, 5 the secondary gene of interest can be used to transform a plant at the same time as the primary gene either by inclusion of both expression constructs in a single transformation vector or by using separate vector, each of which express desired primary or secondary genes. The secondary gene can be introduced into a plant which has already been transformed with the primary gene, or alternatively, transformed 10 plants, one expressing the primary gene and one expressing the secondary gene, can be crossed to bring the genes together in the same plant.

By combining the genes with tissue specific promoters, the carotenoid levels can be altered in particular tissues of the plant. Thus, carotenoid levels in the seed, including embryos and endosperm, can be altered by the use of seed specific 15 transcriptional initiation regions. Such regions are disclosed, for example, in U.S. Patent No. 5,420,034, which disclosure is herein incorporated by reference.

In this manner, the transformed seed provides a factory for the production of modified oils. The modified oil may be used or alternatively, the compounds in the oils can be isolated. Thus, the present invention allows for the production of 20 particular compounds of interest as well as speciality oils.

The primary or secondary genes encoding the enzymes of interest can be used in expression cassettes for expression in the transformed plant tissues. To alter the carotenoid or fatty acid levels in a plant of interest, the plant is transformed with at least one expression cassette comprising a transcriptional initiation region linked to a 25 gene of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions.

The transcriptional initiation may be native or analogous to the host or foreign or heterologous to the host. By foreign is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced.

5 Of particular interest are those transcriptional initiation regions associated with storage proteins, such as napin, cruciferin,  $\beta$ -conglycinin, phaseolin, or the like, and proteins involved in fatty acid biosynthesis, such as acyl carrier protein (ACP). See, U.S. Patent No. 5,420,034, herein incorporated by reference.

10 The transcriptional cassette will include the in 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, 15 such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau et al., (1991), *Mol. Gen. Genet.*, 262:141-144; Proudfoot, (1991), *Cell*, 64:671-674; Sanfacon et al., (1991), *Genes Dev.*, 5:141-149; Mogen et al., (1990), *Plant Cell*, 2:1261-1272; Munroe et al., (1990), *Gene*, 91:151-158; Ballas et al., (1989), *Nucleic Acids Res.*, 17:7891-7903; Joshi et al., (1987), *Nucleic Acid Res.*, 20 15:9627-9639).

For the most part, the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression. Thus, the carotenoid biosynthesis gene or genes of interest may be inserted into the plastid for expression with appropriate plastid constructs and regulatory elements. Alternatively, nuclear 25 transformation may be used in which case the expression cassette will contain a gene encoding a transit peptide to direct the carotenoid biosynthesis gene of interest to the plastid. Such transit peptides are known in the art. See, for example, Von Heijne et al. (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark et al. (1989) *J. Biol. Chem.*

264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun.* 196:1414-1421; and, Shah *et al.* (1986) *Science* 233:478-481. Plant carotenoid genes useful in the invention may utilize native or heterologous transit peptides.

5 It is noted that where the gene or DNA sequence of interest is an antisense DNA, targeting to a plastid is not required. In addition, where antisense inhibition of a given carotenoid biosynthesis gene is desired, the entire DNA sequence derived from the carotenoid biosynthesis gene is not required.

10 The construct may also include any other necessary regulators such as plant translational consensus sequences (Joshi, C.P., (1987), *Nucleic Acids Research*, 15:6643-6653), introns (Luehrs and Walbot, (1991), *Mol. Gen. Genet.*, 225:81-93) and the like, operably linked to the nucleotide sequence of interest.

15 It may be beneficial to include 5' leader sequences in the expression cassette which can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T.R., and Moss, B. (1989) *PNAS USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.*, (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology*, 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak, D.G., and Sarnow, P., (1991), *Nature*, 353:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S.A., and Gehrke, L., (1987), *Nature*, 325:622-625; tobacco mosaic virus leader (TMV), (Gallie, D.R. *et al.*, (1989), *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. *et al.*, (1991), *Virology*, 81:382-385. See also, 20 Della-Cioppa *et al.*, (1987), *Plant Physiology*, 84:965-968.

25 Depending upon where the DNA sequence of interest is to be expressed, it may be desirable to synthesize the sequence with plant preferred codons, or alternatively with chloroplast preferred codons. The plant preferred codons may be

determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest. See, EPA 0359472; EPA 0385962; WO 91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Research* 17: 477-498. In this manner, 5 the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used. For the construction of chloroplast preferred genes, see USPN 5,545,817.

In preparing the transcription cassette, the various DNA fragments may be 10 manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, 15 restriction, annealing, resection, ligation, or the like may be employed, where insertions, deletions or substitutions, *e.g.* transitions and transversions, may be involved.

The recombinant DNA molecules of the invention can be introduced into the 20 plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant, *i.e.* monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) *BioTechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium* 25 mediated transformation (Hinchee *et al.* (1988) *Biotechnology* 6:915-921) and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see, Weissinger *et al.* (1988) *Annual Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37(onion); Christou *et al.* (1988) *Plant Physiol.*

87:671-674(soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740(rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA*, 85:4305-4309(maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444(maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839; and Gordon-Kamm *et al.* (1990) *Plant Cell* 2:603-618 (maize).

5 Alternatively, a plant plastid can be transformed directly. Stable transformation of chloroplasts has been reported in higher plants, see, for example, Svab *et al.* (1990) *Proc. Nat'l. Acad. Sci. USA* 87:8526-8530; Svab & Maliga (1993) *Proc. Nat'l Acad. Sci. USA* 90:913-917; Staub & Maliga (1993) *Embo J.* 12:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. In such methods, plastid gene expression can be accomplished by use of a plastid gene promoter or by trans-activation of a silent plastid-borne transgene positioned for 10 expression from a selective promoter sequence such as that recognized by T7 RNA polymerase. The silent plastid gene is activated by expression of the specific RNA polymerase from a nuclear expression construct and targeting of the polymerase to the plastid by use of a transit peptide. Tissue-specific expression may be obtained in such a method by use of a nuclear-encoded and plastid-directed specific RNA polymerase 15 expressed from a suitable plant tissue specific promoter. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci., USA* 91:7301-7305.

20

25 The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.*, *Plant Cell Reports* (1986), 5:81-84. These plants may then be grown, and either self or crossed with a different plant strain, and the resulting homozygotes or hybrids having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited

and then seeds harvested to ensure the desired phenotype or other property has been achieved.

As a host cell, any plant variety may be employed. Of particular interest, are plant species which provide seeds of interest. For the most part, plants will be chosen 5 where the seed is produced in high amounts, a seed-specific product of interest is involved, or the seed or a seed part is edible. Seeds of interest include the oil seeds, such as oilseed *Brassica* seeds, cotton seeds, soybean, safflower, sunflower, coconut, palm, and the like; grain seeds, e.g. wheat, barley, oats, amaranth, flax, rye, triticale, rice, corn, etc.; other edible seeds or seeds with edible parts including pumpkin, 10 squash, sesame, poppy, grape, mung beans, peanut, peas, beans, radish, alfalfa, cocoa, coffee, tree nuts such as walnuts, almonds, pecans, chick-peas etc.

It is noted that the methods of the present invention have been demonstrated to provide increased carotenoid production in both oilseed *Brassica*, which has a green embryo, and in cotton, which has a white embryo.

15 In seed of cotton plants transformed with phytoene synthase, increases of total carotenoid levels ranging from 10 to 300 fold may be obtained. The majority of the increase in carotenoid levels, in this case, about 80%, is observed as an increase in phytoene levels. Increases in lutein levels are also obtained in this case, ranging from 1.5 to a 5 fold increase. In addition,  $\alpha$ -carotene and  $\beta$ -carotene levels are also 20 increased 10 to 100 fold, with  $\beta$ -carotene levels being 20 fold higher than  $\alpha$ -carotene levels. Thus, as seen with *Brassica*, a second early carotenoid biosynthesis gene, such as phytoene desaturase, may be used with *crtB* to increase the metabolic flux through the carotenoid/ isoprenoid pathway in cotton to produce a particular carotenoid.

Furthermore, it should also be noted that the methods of the present invention 25 have also been demonstrated herein to provide increased carotenoid production in additional plant species, such as *Arabidopsis*.

In seed of *Arabidopsis* plants transformed with phytoene synthase, increases of total carotenoid levels ranging from 3 to at least approximately 20 fold may be

obtained. A large increase in the level of  $\beta$ -carotene, ranging from 10 to 70 fold, are observed in seeds of transgenic *Arabidopsis* plants. Increases in lutein levels are also obtained in this case, ranging from 1.5 to a 3 fold increase. In addition, phytoene,  $\alpha$ -carotene and lycopene levels are also increased. However, such increases in  $\alpha$ -carotene, phytoene and lycopene are difficult to quantify as these levels are too low to measure in nontransformed control plants. Thus, as seen with *Brassica* and cotton, a second early carotenoid biosynthesis gene may be used with *crtB* to increase the metabolic flux through the carotenoid/ isoprenoid pathway in cotton to produce a particular carotenoid and to reduce the increased levels of phytoene.

10 In one embodiment of the invention, seed transcriptional initiation regions are used in combination with at least one carotenoid biosynthesis gene. This increases the activity of the carotenoid pathway and alters carotenoid levels in the transformed seed. In this manner, particular genes can be selected to promote the formation of compounds of interest. Where the gene selected is an early carotenoid biosynthesis 15 gene the transformed seed has a significant increase in carotenoid biosynthesis as the result of an increase in the flux through the pathway. For *Brassica* seeds transformed with an early carotenoid biosynthesis gene, significant increases in the production of  $\alpha$ -carotene,  $\beta$ -carotene and smaller increases in lutein in the seed oil, as well as altered oil fatty acid compositions are obtained.

20 Where the early carotenoid biosynthesis gene is phytoene synthase, significant increases of a particular carotenoid include those ranging from a 10 to a 50 fold increase, preferably at least a 50 to a 100 fold increase, more preferably, at least a 50 to a 200 fold increase, such as the increases seen in  $\alpha$ -carotene and  $\beta$ -carotene levels. Lutein levels, in this case, are also increased, but lower increases of 1.5 - 2 fold are 25 obtained. At the same time, total carotenoid levels may be increased at least 10 to 25 fold, preferably 25 to 60 fold, and more preferably 25 to 100 fold. Thus, a seed of the invention transformed with a phytoene synthase gene has a substantial increase in levels of  $\alpha$ - and  $\beta$ -carotene and total carotenoids, as well as smaller increases in lutein

and other carotenoids, including phytoene. In some cases, it is not possible to quantitate the fold increase in a given carotenoid compound, as the levels are too low to detect in seeds from comparable non-transformed plants. In *Brassica napus*, for example,  $\alpha$ -cryptoxanthin, lycopene, phytoene and phytofluene are all detected in various levels in seeds transformed with a *crtB* gene, but are not detectable in seeds from untransformed *Brassica napus* plants.

Where the early carotenoid biosynthesis gene is GGPP synthase or phytoene desaturase, 1.5 to 2 fold increases in lutein and  $\beta$ -carotene have been obtained in at least one transgenic plant for each gene. Lycopene is also detected in seeds from *Brassica napus* plants transformed with a *crtE* (GGPP synthase) gene. Total carotenoids in *crtE* or *crtI* transformants are also increased approximately 2 fold. Chlorophyll levels are also increased in *B. napus* transgenic plants expressing a *crtE* gene suggesting an increase in the levels of geranylgeranyl pyrophosphate (GGPP), which is the branch point substrate for carotenoid, chlorophyll and tocopherol biosynthesis. Increases in chlorophyll levels of 1.5 to 2 fold may be obtained in developing and mature seeds. Thus, also of interest as sources of carotenoids are plants which have been engineered to express increased levels of both *crtB* and *crtE*.

As demonstrated herein, the effect of one early carotenoid biosynthesis gene on the metabolic energy flux through the carotenoid pathway may be further effected by the addition of a second early carotenoid biosynthesis gene. Thus, the addition of a second early carotenoid biosynthesis gene for increasing the metabolic flow through the carotenoid biosynthesis pathway is also of interest in the present invention, and may find use for production of particular carotenoids either in the presence or absence of a secondary carotenoid biosynthesis gene.

Where the early carotenoid biosynthesis gene phytoene synthase is cotransformed into *Brassica napus* with a second early carotenoid biosynthesis gene, phytoene desaturase, significant increases of particular carotenoids include increases in  $\alpha$ -carotene,  $\beta$ -carotene, and lutein such as observed by expression of *crtB* alone. In

addition, lycopene and phytoene levels are also increased in such plants, but increases are difficult to quantitate as these levels are too low to be detected in untransformed *Brassica napus* plants.

Furthermore, when *crtI* and *crtB* are both expressed, total carotenoid levels 5 greater than those observed with *crtB* alone may be obtained. In at least one plant, total carotenoid levels of 1.5 fold those observed in *crtB* plants were obtained. Lycopene levels are also increased over levels obtained in seeds of plants transformed with *crtB* alone. Lycopene levels may be increased from 4 to 15 fold over those obtained in seed of a homozygous *crtB* plant. In addition, a reduction in the ratio of 10 phytoene to total carotenoids is also obtained, and as a result, levels of  $\alpha$ -carotene and  $\beta$ -carotene are increased 1.2 to 1.8 fold over those obtained with *crtB* alone. In seeds of plants transformed with phytoene synthase alone, phytoene levels constituted as 15 much as 20% of total carotenoids, while in plants cotransformed with phytoene synthase and phytoene desaturase, phytoene levels represent only 4% to 7% of the total carotenoids.

This metabolic energy effected by transformation with an early carotenoid gene can be funneled into a metabolic compound of choice by transformation with a second gene. As discussed above, the second gene is designed to promote the synthesis of a particular carotenoid by promoting the formation of the carotenoid of 20 interest or alternatively by stopping the pathway to allow for the buildup of compounds. Therefore, significant amounts of carotenoids of interest can be produced in the transformed seeds of the present invention.

Where the primary carotenoid biosynthesis gene phytoene synthase is cotransformed with a secondary carotenoid biosynthesis gene,  $\beta$ -carotene ketolase, 25 increases in levels of  $\alpha$ -carotene,  $\beta$ -carotene and phytoene, such as those seen with transformation with *crtB* alone, are obtained. Furthermore, echinenone and canthaxanthin levels are also increased. However, such increases are difficult to quantitate as echinenone and canthaxanthin are either not produced in *Brassica napus*

, or the levels are too low to be detected in *B. napus* plants expressing phytoene synthase alone and nontransformed control plants. Thus, for the production of a specific carotenoid, such as astaxanthin, the addition of a third carotenoid biosynthesis gene, such as  $\beta$ -carotene hydroxylase (*crtZ*), may find use in the present invention.

5 Furthermore, the addition of a fourth carotenoid biosynthesis gene, such as phytoene desaturase, may also find use in the present invention.

It should be noted that the carotenoid echinenone is a reaction intermediate in the production of canthaxanthin from  $\beta$ -carotene. The  $\beta$ -carotene ketolase (*crtW*) could react with the  $\beta$ -ring of  $\alpha$ - or  $\beta$ -carotene. One  $\beta$ -ring reaction in  $\beta$ -carotene 10 results in echinenone, two  $\beta$ -ring reactions in  $\beta$ -carotene form canthaxanthin, and one  $\beta$ -ring reaction in  $\alpha$ -carotene makes 4-keto- $\alpha$ -carotene. This enzyme can not react with the  $\epsilon$ -ring of  $\alpha$ -carotene. Thus, two additional peaks on the HPLC chromatogram are produced in similar amounts, one representing echinenone, and the other may represent 4-keto- $\alpha$ -carotene.

15 Where the primary carotenoid biosynthesis gene phytoene synthase is cotransformed with an antisense secondary carotenoid biosynthesis gene,  $\epsilon$ -cyclase, large increases in levels of  $\alpha$ -carotene,  $\beta$ -carotene and phytoene, such as those seen with transformation with *crtB* alone, are obtained. Some difference in the ratio of  $\beta$ -carotene to  $\alpha$ -carotene is observed as compared to plants transformed with *crtB* alone, 20 but large increases in both  $\alpha$ -carotene and  $\beta$ -carotene levels are still observed. Lutein levels, on the other hand, are either unchanged, increased, or in some cases decreased by as much as 80% as compared to seeds of untransformed control plants.

Initiation of carotenoid biosynthesis begins at approximately 15 days post 25 anthesis in *B. napus* seeds, while expression of transformed genes utilizing the napin promoter begins about 18 days post anthesis. Thus, in order to more tightly control the  $\alpha$ -carotene pathway to allow for the build up of  $\beta$ -carotene pathway carotenoids using antisense  $\epsilon$ -cyclase, an earlier promoter, such as that of the *Lesquerella kappa* hydroxylase (described in pending U.S. patent application 08/898,038, filed 18 July,

1997), may find use. Thus, for increasing levels of a particular carotenoid using antisense, an earlier seed specific transcriptional initiation region, may be used with a secondary carotenoid biosynthesis gene.

The seeds of the invention which have been transformed with the primary 5 early carotenoid biosynthesis gene also provide a source for novel oil compositions. The use of phytoene synthase as the primary gene, for example, results in substantial increases in oleic acid content in seed oil. By substantial increase is intended an increase of from about 5% to about 40%, specifically from about 20% to about 40%, more specifically from about 30% to about 40%. Thus, the seeds of the invention 10 which have been transformed with a primary early carotenoid biosynthesis gene provide a source for modified oils having a high oleic acid content. That is, carotenoid biosynthesis genes, particularly early carotenoid biosynthesis genes can be used to produce seeds having at least 70% oleic acid, on a weight percentage basis. The oleic acid content in any seed can be altered by the present methods, even those 15 seeds having a naturally high oleic acid content. Alteration of seeds having naturally high oleic acid contents by the present methods can result in total oleic acid contents of as high as 80%.

Importantly, there is also a decrease in linoleic and linolenic acid content. By 20 decrease in linoleic fatty acid content is intended a decrease from about 10% to about 25%, preferably about 25% to about 40%, more preferably about 35% to about 60%. By decrease in linolenic fatty acid content is intended a decrease from about 10% to about 30%, preferably about 30% to about 60%, more preferably about 50% to about 75%. Thus, the methods of the invention result in oils which are more oxidatively 25 stable than the naturally occurring oils. The modified oils of the invention are low-saturate, high oleic and low linolenic. Furthermore, the present invention provides oils high in monounsaturated fatty acids which are important as a dietary constituent.

Based on the methods disclosed herein, seed oil can be modified to engineer an oil with a high oleic acid content as well as a high level of a carotenoid of interest.

High oleic acid and high  $\alpha$ - and  $\beta$ -carotene oils would have a longer shelf life as both the oleic acid and  $\alpha$ - and  $\beta$ -carotene content would lend stability. It is also noted that such oils are more desirable as sources of carotenoids than the natural red palm oil, which oil contains high levels of saturated fatty acids.

5 The transformed seed of the invention can thus provide a source of carotenoid products as well as modified fatty acids. Where the intent is to produce particular carotenoid compounds of interest, methods are available in the art for the purification of the carotenoid compounds. In the same manner, methods available in the art can be utilized to produce oils purified of carotenoids. See, generally, WO 96/13149 and  
10 Favati *et al.* (1988) *J. Food Sci.* 53:1532 and the references cited therein.

The transformed seed and embryos additionally find use as screenable markers. That is, transformed seed and embryos can be visually determined and selected based on color as a result of the increased carotenoid content. The transformed seeds or embryos display a color ranging from yellow to orange to red as  
15 a result of the increased carotenoid levels. Therefore, where plant transformation methods involve an embryonic stage, such as in transformation of cotton or soybean, the carotenoid gene can be used in plant transformation experiments as a marker gene to allow for visual selection of transformants. Likewise, segregating seed can easily be identified as described further in the examples.

20 The following examples are offered by way of illustration and not by way of limitation.

## EXPERIMENTAL

### Example 1 Expression Construct and Plant Transformation

#### 25 A. SSU fusions to *E. uredovora* carotenoid biosynthesis genes

##### (1) Phytoene Synthase

The SSU leader and *crtB* gene sequences were joined by PCR. The sequence of the SSU/*crtB* fusion is shown in Figure 1. The *crtB* gene from nucleotides 5057 to

5363 (numbering according to Misawa *et al.* (1990) *supra*) was joined to the SSU leader as follows. A *Bg*III site was included upstream of the SSU leader start site to facilitate cloning. The thymidine nucleotide at 5057 of *crtB* was changed to an adenine to make the first amino acid at the SSU leader/*crtB* junction a methionine, 5 and the splice site a cys-met-asn. The native splice site for SSU is cys-met-gln. Note that Misawa *et al.* (1990) *supra*) indicates that the start site for the coding region for *crtB* is at nucleotide 5096. Thus, there are 13 amino acids upstream of the published start of the coding region for *crtB* and after the SSU splice site in the *crtB*/SSU fusion. Twelve of these amino acids are translated from *Erwinia* *crtB* upstream 10 sequence and one is the added methionine. The *crtB* from 5363 (*EcoRV*) to 6009 (*EcoRI*) was then attached to the SSU-*crtB* fusion to obtain a complete SSU-*crtB* fusion construct designated pCGN3373 (Fig. 1).

(2) Phytoene Desaturase

15 A plasmid comprising a *E. uredovora* *crtI* gene fused to the transit peptide sequence of the pea Rubisco small subunit was described by Misawa *et al.* (*The Plant Journal* (1993) 4:833-840. An approximately 2.1 kb *Xba*I/*Eco*RI fragment of this plasmid containing the SSU-*crtI* fusion and a nos 3' termination region was cloned in position for expression from a napin 5' promoter.

(3) GGPP Synthase

20 A similar construct containing the SSU transit fused to an *E. uredovora* *crtE* gene was obtained. The SSU-*crtE* fusion is present on an approximately 1.2 kb *Bg*III/*Bam*HI fragment in pCGN3360.

B. SSU fusions to *A. aurantiacum* carotenoid biosynthesis genes

## (4) beta-Carotene Hydroxylase (crtZ)

The SSU leader and *crtZ* gene sequences were joined by PCR. The *crtZ* gene (Misawa, *et al.* (1995) *supra*) nucleotide sequence was resynthesized to adjust for plant codon usage. The re synthesized *crtZ* gene was joined to the SSU leader by PCR as follows. A *Bgl*II site was included upstream of the SSU leader translation start site and a *Xho*I site was included downstream of the *crtZ* stop codon to facilitate cloning in the napin expression cassette. The nucleotide sequence of the complete *ssu:crtZ* fusion is shown in Figure 15.

## 10 (5) beta-Carotene Ketolase (crtW)

The SSU leader and *crtW* gene sequences were joined by PCR. The *crtW* gene (Misawa, *et al.* (1995) *supra*) nucleotide sequence was resynthesized to adjust for plant codon usage. The re synthesized *crtW* gene was joined to the SSU leader by PCR as follows. A *Bgl*II site was included upstream of the SSU leader translation start site and a *Xho*I site was included downstream of the stop codon to facilitate cloning in the napin expression cassette. The nucleotide sequence of the complete *ssu:crtW* fusion is shown in Figure 16.

20

C. Expression Constructs for Plant Transformation

## (1) Phytoene Synthase

pCGN3373 carrying the complete SSU/*crtB* fusion was cut with *Bgl*II and *Bam*HI to excise the SSU/*crtB* fusion. The resulting fragment was ligated into the napin expression cassette in pCGN3223 at the *Bam*HI site (see WO 94/10288 for description of napin expression cassette). The resulting construct, pCGN3389, was digested with *Hind*III to excise the napin 5'-SSU/*crtB*-napin 3' fragment, which was then cloned into *Hind*III cut pCGN1559PASS yielding pCGN3390. pCGN1559PASS is a binary vector for *Agrobacterium*-mediated transformation such as those described

by McBride *et al.* (*Plant Mol. Biol.* (1990) 14:269-276) and is prepared from pCGN1559 by substitution of the pCGN1559 linker region with a linker region containing the following restriction digestion sites: *Asp718/AscI/PacI/XbaI/BamHI/SwaI/Sse8387(PstI)/HindIII*. A map of pCGN3390 is provided in Figure 2A.

5 (2) Phytoene Desaturase

A fragment comprising a napin 5'/SSU-*crtI* fusion/nos 3' construct as described above was cloned into a binary vector for plant transformation resulting in pCGN9010. A map of pCGN9010 is provided in Figure 2C.

(3) GGPP Synthase

10 pCGN3360 carrying the complete SSU-*crtE* fusion was cut with *BglII* and *BamHI* to excise the SSU-*crtE* fusion. The resulting 1.2 kb fragment was ligated into the napin expression cassette in pCGN3223 at the *BamHI* site. The resulting construct, pCGN3391, was digested with *HindIII* to excise the napin promoter-SSU-*crtE* napin 3' fragment, which was then cloned into *HindIII* cut pCGN1559PASS yielding pCGN3392. A map of pCGN3392 is provided in Figure 2B.

15 (4) Phytoene Synthase + Phytoene Desaturase

The napin 5'-SSU-*crtB*-napin 3' fragment from pCGN3389 and the napin 5'/SSU-*crtI* fusion/nos 3' as present in pCGN9010 were inserted into a binary vector resulting in pCGN9009, shown in Figure 2D.

20 (5) Antisense Epsilon Cyclase + Phytoene Synthase

*Brassica napus* epsilon cyclase genes are isolated by PCR using primers designed from an *Arabidopsis* epsilon cyclase gene (Cunningham FX Jr (1996) *Plant Cell* 8:1613-1626). Sequence of *B. napus* epsilon cyclase genes is provided in Figures 9 (clone 9-4) and 10 (clone 7-6). An antisense construct is prepared by 25 cloning an *XhoI/BamHI* fragment of cDNA clone 9-4 into a napin expression cassette (pCGN3223) digested with *XhoI* and *BglII*. The napin 5'-antisense epsilon cyclase-napin 3' fragment is cloned along with a napin 5'-SSU-*crtB*-napin 3' fragment,

fragment into a binary vector for plant transformation, resulting in pCGN9002, shown in Figure 2E.

(6) Antisense Beta Cyclase + Phytoene Synthase

Brassica napus beta cyclase genes are isolated by PCR using primers designed 5 from an *Arabidopsis* beta cyclase gene (Cunningham FX Jr (1996) *Plant Cell* 8:1613-1626). Sequence of a *B. napus* beta cyclase cDNA, 32-3, is provided in Figures 11. An antisense construct is prepared by cloning an *Xho*I fragment of the beta cyclase cDNA clone into a napin expression cassette (pCGN3223) digested with *Xho*I. A clone containing the beta cyclase in the antisense orientation is selected. The 10 napin 5'-antisense beta cyclase-napin 3' fragment is cloned along with a napin 5'-SSU/*crtB*-napin 3' fragment into a binary vector for plant transformation, resulting in pCGN9017, shown in Figure 2F.

(7) beta-Carotene Hydroxylase + Phytoene synthase

The vector pCGN9003 was constructed by removing the restriction sites 15 between the *crtB* coding sequence and the napin 3' sequence by digestion with *Clal* and *Xho*I and filling the ends with klenow creating the vector pCGN9000. PCGN9000 was digested with *Asp*718, and the fragment containing the napin 5'/SSU:*crtB*/napin 3' was ligated into the binary vector pCGN5139.

A binary vector for plant transformation, pCGN5139, was constructed 20 using the neomycin phospho-transferase (nptII) kanamycin resistance gene driven by the CAMV 35S transcriptional initiation region (35S 5') and transcription termination (35S 3') sequences (Fraley et al., *Proc. Natl. Acad. Sci* (1983) 80:4803-4807, Gardner et al., (1986) *Plant Mol Biol* 6:221-228). The 35S 5'-nptII-35S 3' fragment was then cloned into a vector containing ori322, Right border (0.5Kb), lacZ, Left Border 25 (0.58Kb), as an *Xho* I fragment between the Right border-lacZ and Left border sequences. The ColEI and pRi origins of replication as well as the Gentamycin resistance gene were aquired from a derivative of pCGN1532 (McBride and Summerfelt, *Plant Molecular Biology*, (1990), 14:269-276). Finally, a linker

containing unique restriction sites was synthesized and cloned into the *Asp 718/Hind III* (within the *lacZ* sequence) sites to create the binary vector pCGN5139.

The plastid targeted *ssu:crtZ* fusion was cloned into the napin pCGN3223 seed expression cassette as a *Bgl II-Xho I* fragment to generate pCGN6203. The plasmid 5 pCGN6203 carrying the complete napin cassette with *ssu:crtZ* was digested with *NotI* to excise the napin cassette containing the *ssu:crtZ* coding region. The excised fragment was ligated into the *Not I* site of the binary pCGN9003 carrying the napin SSU:crtB construct. The resulting construct, pCGN6205 (Figure 2H) is a binary vector for Agrobacterium-mediated transformation such as those described 10 by McBride et al. (Plant Mol. Biol. (1990) 14:269-276) and is prepared from pCGN1559 by substitution of the pCGN1559 linker region with a linker region containing the following restriction digestion sites: *Asp718/AscI/PacI/XbaI/BamHI/SwaI/Sse8387(PstI)/HindIII*. A map of pCGN6205 is provided in Figure 2H.

15 (8) *beta-Carotene Ketolase + Phytoene synthase*

The *ssu crtW* plastid targeted fusion was cloned into the napin pCGN3223 seed expression cassette as a *Bgl II-Xho I* fragment to generate plasmid pCGN6202.

The plasmid pCGN6202 carrying the napin cassette with *ssu:crtW* was digested with *NotI* to excise a DNA fragment containing the napin cassette with 20 *ssu:crtZ*. The resulting fragment was ligated into the *Not I* site of the binary pCGN9003 (described above) carrying the SSU:crtB napin construct. The resulting pCGN6204 (Figure 2G) is a binary vector for Agrobacterium-mediated transformation such as those described by McBride et al. (Plant Mol. Biol. (1990) 14:269-276) and is prepared from pCGN1559 by substitution of the pCGN1559 25 linker region with a linker region containing the following restriction digestion sites: *Asp718/AscI/PacI/XbaI/BamHI/SwaI/Sse8387(PstI)/HindIII*. A map of pCGN6204 is provided in Figure 2G.

(9) *Phytoene synthase+ beta-Carotene hydroxylase+ beta-Carotene Ketolase*

Construct pCGN6203 containing the napin cassette and ssu:crtZ was digested with Hind III to excise the fragment containing napin ssu:crtZ. The resulting HindIII fragment was cloned into the Hind III site of pCGN6204 to generate a triple crt genes binary pCGN6206 that contains napin ssu:crtB+ napin ssu:crtW+ napinssu:crtZ (Figure 2I).

**D. Plant Transformation**

Transformed *Brassica napus* plants containing the above described constructs are obtained as described in Radke *et al.* (*Theor. Appl. Genet.* (1988) 75:685-694 and *Plant Cell Reports* (1992) 11:499-505).

10 Transformed cotton plants, *Gossypium hirsutum*, containing phytoene synthase may be obtained using methods described in issued U.S. patent No. 5,004,863, and 5,159,135, and in Umbeck *et al.* (1987) *Bio/Technology* 5:263-266, or as described in copending application 08/539,176.

15 Transgenic *Arabidopsis thaliana* plants containing phytoene synthase may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) 85:5536-5540), or as described by Bent *et al.* ((1994), *Science* 265:1856-1860), or Bechtold *et al.* ((1993), *C.R.Acad.Sci, Life Sciences* 316:1194-1199).

20 **Example 2 Analysis of Transgenic Plants**

**A. Visual Observations and Segregation Ratios**

25 The napin-SSU leader/crtB plants in 212/86 were tagged at 21 days, 28 days and 35 days post anthesis. When the first plant, 3390-1 was harvested at 28 days, some of the seeds were obviously orange. AT 35dpa, the orange was obvious enough that a segregation ratio could be obtained. This trend of orange seeds has continued and is seen in each of the 17 lines harvested that have been obtained. A table of the segregation ratios is included below in Table 3.

TABLE 3

	<b>Generation</b>	<b>Plant #</b>	<b>Orange</b>	<b>Green</b>	<b>Ratio</b>	<b>Chi Square</b>
5	T2	3390-1	291	88	3 to 1	0.64
	T2	3390-2	150	22	No fit	
	T2	3390-8	293	87	3 to 1	0.90
	T2	3390-4	277	82	3 to 1	0.89
	T2	3390-5	243	62	3 to 1	1.90
10	T2	3390-7	236	89	3 to 1	0.99
	T2	3390-6	307	5	63 to 1	0.00
	T2	3390-3	121	50	No fit	1.64
	T2	3390-11	294	105	3 to 1	0.37
	T2	3390-15	287	83	3 to 1	1.30
15	T2	3390-16	187	65	3 to 1	0.08
	T2	3390-17	105	104	No fit	
	T2	3390-12	119	28	3 to 1	2.78
	T2	3390-14	283	107	3 to 1	1.23
	T2	3390-19	238	94	3 to 1	1.94
20	T2	3390-20	251	4	63 to 1	0.00
	T2	3390-27	229	4	63 to 1	0.04

B. Carotenoid Analysis of Developing Seeds

Carotenoids were extracted from seeds harvested at approximately 35 days post-anthesis as follows. Eight seed samples of orange seeds from transgenic plant 25 3390-1 and eight seed samples of a 212/86 variety rapeseed control plant were ground in 200 $\mu$ l of 70% acetone/30% methanol. The ground seed mixture was then spun in a microcentrifuge for approximately 5 minutes and the supernatant removed. Two additional 70% acetone/30% methanol extractions were conducted with the pelleted seed material and all three supernatants pooled and labeled A/M extract.

30 At this point in the extraction, the control seed pellets are white, whereas the seed pellets from the transgenic seeds have a yellow color. The pellets are then extracted twice with ether and the resultant supernatants pooled and labeled E extract. The A/M extract was then transferred to ether as follows. 450 $\mu$ l ether and 600 $\mu$ l of

water were added to the extracts, followed by removal of the ether layers. The A/M extracts were then washed two more time with 400 $\mu$ l of ether, and the ether fractions from the three A/M washes pooled. The E extracts described above were washed once with 400 $\mu$ l of water and pooled with the A/M ether fractions. The pooled ether 5 fractions were blown down to a volume of approximately 300 $\mu$ l with nitrogen gas and filtered using a syringe microfilter. The sample vials were rinsed with approximately 100 $\mu$ l ether and the rinse was similarly filtered and pooled with the initial filtrate, yielding total volume of approximately 150 $\mu$ l. A 50 $\mu$ l aliquot was stored at -20°C until further analysis and the remaining 100 $\mu$ l sample was saponified as follows.

10 100 $\mu$ l of 10% potassium hydroxide (KOH) in methanol was added to each 100 $\mu$ l sample and the mixture stored in the dark at room temperature for approximately 2 hours. 400 $\mu$ l of water was then added to the samples and the ether phase removed. For better phase separation, saturated NaCl may be substituted for the water. The water solution was then extracted twice more with 100 $\mu$ l of ether and the ether 15 samples pooled and washed with water.

The saponified samples were then analyzed by HPLC analysis on a Rainin microsorb C18 column (25cm length, 4.6mm outside diameter) at a flow rate of 1.5ml per minute. The gradient used for elution is as follows:

20 A = acetonitrile  
B = hexane/methylene chloride (1:1)  
C = methanol.

The initial solution was 70:20:10 (A:B:C). At 2.5 minutes the solution is ramped over 5 minutes to 65:25:10 (A:B:C) and held at this for 12.5 minutes. The solution is then ramped to 70:20:10 (A:B:C) over two minutes followed by a three minute delay prior 25 to injection of the next sample. The absorbance of the eluting samples is continuously monitored at 450 and 280 nm and known chemical and biological standards were used to identify the various absorbance peaks.

In Figures 3 and 4, results of analyses of saponified samples are provided for control and pCGN3390 transformed seeds, respectively. Clear increases in the levels of  $\alpha$ - and  $\beta$ -carotene and phytoene in the transgenic plant seeds are observed, as well as smaller increases in levels of the hydroxylated carotenoid, lutein.

5      C. Carotenoid and Tocopherol Analysis of Mature Seeds from *crtB* Transgenic Plants

Mature 3390 T2 seed were sent to an analytical laboratory for quantitative analysis using standard HPLC methods known in the art. These results of these analysis are shown in Table 4 below. Compound levels are presented as  $\mu\text{g/g}$ .

Seeds designated "Maroon" were selected based on seed color. The seeds  
10     which have orange embryos appear maroon colored at maturity as opposed to the black-brown appearance of seeds from wild type plants of this cultivar. Seeds  
designated as "Random" were not selected for color. As 3390-1 is segregating 3 to 1  
for Kan, the "Random" population includes a proportion of nulls. The maroon  
population contains only transgenics. Due to an effort to exclude nulls from this  
15     population, the inclusion of homozygotes may be favored.

TABLE 4

	COMPOUND	CONTROL	3390-1 RANDOM	3390-1 MAROON
5	Lutein	7.2	18	26
	Zeaxanthin	nd*	nd	nd
	$\alpha$ -cryptoxanthin	nd	8	15
	$\beta$ -cryptoxanthin	nd	nd	nd
	Lycopene	nd	2.3	5.1
10	cis-Lycopene	nd	2.9	5.4
	$\alpha$ -carotene	0.6	124	244
	$\beta$ -carotene	0.9	177	338
	cis- $\beta$ -carotene	0.2	12	26
	Other	6	34	51
15	Total colored carotenoids	14.9	378.2	710.5
	Phytoene	nd	62	139
	Phytofluene	nd	24	54
	Total all carotenoids	14.9	464.2	903.5
20	Alpha-tocopherol	74	93	109
	Gamma-tocopherol	246	188	95
	Delta-tocopherol	3	5	5

\*nd = not detected

25

In the non-transgenic sample, "other" includes mostly very polar compounds, such as neoxanthin, violaxanthin, etc. In the transgenic sample "other" includes these and additional compounds, such as zeta-carotene, neurosporene, and mono-cyclic carotenoids.

30 Results of carotenoid analysis of 3390 T2 seeds from transformed plants of *B. napus* variety Quantum (SP30021) are presented in Figure 12.

Results of carotenoid analysis of 3390 T3 seeds from transformed plants of *B. napus* variety 212/86 (SP001) are presented in Figure 13.

The above results demonstrate that  $\alpha$ - and  $\beta$ -carotenes levels are significantly increased in the mature seeds as the result of expression of the *crtB* gene. Generally, the overall increase in carotenoids is quite high, nearly 50 fold for colored carotenoids and up to 60 fold if phytoene and phytofluene are included. It is clear that the flux 5 through the isoprenoid pathway has been dramatically increased. Additionally it is noted that the  $\alpha$ -tocopherol (Vitamin E) levels are also increased by nearly 50%.

#### D. Germination Studies

Ten mature seeds of 3390-1 and 10 seeds of 212/86 control were planted in soil and grown in a walk-in growth chamber. The transgenics emerged 1 to 2 days 10 later than the controls, however, all 10 seeds did germinate. The transgenics were yellowish-pink when they first emerged but greened up in one to two days. At the emergence of the first true leaf, no difference in color was observed. Plants germinated from both the transgenic and control seeds developed normally.

#### E. Fatty Acid Analysis

15 Fatty acid composition of mature seeds was determined by GC analysis of single T2 seeds harvested from transgenic plants 3390-1 and 3390-8. Single seeds from both Random (R) and Maroon (M) populations (as defined above) were analyzed and compared to seeds from a 212/86 control (SP001-1). The results of these analyses are provided in Table 5 below as weight % total fatty acids.

**TABLE 5**  
**FATTY ACID COMPOSITION OF 3390-1 AND 3390-8 LINES**

SAMPLE	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
CONTROL	1.5	0	0.1	5.1	0.4	1.7	59.9	17.1	12.0	0.6	1.2	0.1	0.3
CONTROL	1.8	0	0.1	5.1	0.4	1.7	60.1	16.6	12.1	0.6	1.2	0.1	0.3
CONTROL	2.0	0	0.1	5.0	0.4	1.6	60.5	16.2	12.0	0.6	1.2	0.1	0.3
CONTROL	2.2	0	0.1	5.2	0.4	1.6	57.2	18.2	12.7	0.6	1.3	0.1	0.4
CONTROL	1.6	0	0.1	4.7	0.4	1.8	62.7	15.3	11.3	0.6	1.2	0.1	0.3
3390-1-R	2.8	0	0.1	4.8	0.5	3.6	69.9	10.6	4.8	1.2	1.1	0.0	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.5	58.1	19.3	12.3	0.5	1.2	0.1	0.3
3390-1-R	3.5	0	0.1	4.2	0.3	2.6	71.1	9.6	5.8	1.0	1.2	0.0	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.9	61.0	17.8	10.4	0.7	1.3	0.1	0.3
3390-1-R	2.2	0	0.1	4.4	0.3	3.1	73.6	8.9	4.4	1.2	1.1	0.0	0.7
3390-1-R	1.9	0	0.1	4.5	0.3	2.4	72.7	10.6	4.7	0.9	1.3	0.1	0.6
3390-1-R	2.5	0	0.1	4.2	0.3	3.4	71.7	10.0	5.1	1.1	1.0	0.0	0.6
3390-1-R	1.7	0	0.1	4.4	0.3	2.6	73.5	10.0	4.5	1.0	1.2	0.1	0.6
3390-1-R	1.9	0	0.1	4.2	0.3	2.3	72.4	9.9	6.3	0.9	1.2	0.1	0.5
3390-1-R	2.5	0	0.1	4.2	0.3	2.7	72.0	10.1	5.1	1.0	1.2	0.1	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.7	58.5	18.5	12.6	0.6	1.2	0.1	0.3
3390-1-R	2.8	0	0.1	4.6	0.4	3.7	71.8	9.1	4.2	1.3	1.2	0.0	0.7
3390-1-R	1.8	0	0.1	4.0	0.3	2.3	72.4	11.1	5.2	0.9	1.3	0.1	0.5
3390-1-R	1.7	0	0.1	4.4	0.3	2.7	73.9	9.9	4.2	1.0	1.2	0.1	0.6
3390-1-R	1.7	0	0.1	4.6	0.4	2.6	71.4	10.9	5.5	1.0	1.3	0.1	0.6
3390-1-R	2.7	0	0.1	4.2	0.3	2.8	72.1	9.9	5.0	1.1	1.3	0.0	0.6
3390-1-R	2.0	0	0.1	4.5	0.3	3.0	72.5	9.7	4.6	1.2	1.3	0.1	0.7
3390-1-R	1.8	0	0.1	4.9	0.4	3.4	71.8	10.4	4.2	1.2	1.2	0.0	0.7
3390-1-R*	0.9	0	0.1	4.5	0.3	1.7	55.9	18.8	15.6	0.5	1.3	0.1	0.3
3390-1-R*	1.4	0	0.1	4.8	0.4	1.7	57.1	18.0	14.4	0.6	1.2	0.1	0.3
3390-1-R*	1.4	0	0.1	4.5	0.3	1.7	57.8	18.5	13.5	0.6	1.3	0.1	0.3
3390-1-R	2.2	0	0.1	4.5	0.3	2.5	73.4	9.7	4.6	0.9	1.2	0.0	0.5
3390-1-R	1.5	0	0.1	3.8	0.3	2.7	75.9	8.1	4.6	1.0	1.4	0.0	0.6
3390-1-R	1.6	0	0.1	4.5	0.3	2.6	71.9	10.6	5.5	1.0	1.3	0.1	0.6
3390-1-R*	1.3	0	0.1	6.2	0.5	1.4	53.6	21.7	13.2	0.5	1.1	0.1	0.3
3390-1-R	2.1	0	0.1	4.3	0.3	2.4	72.3	10.7	5.1	0.9	1.2	0.0	0.6
3390-1-R*	1.3	0	0.1	5.0	0.3	1.6	57.8	18.8	13.0	0.5	1.3	0.1	0.3
3390-1-R	2.1	0	0.1	4.4	0.3	3.3	72.7	9.2	4.8	1.2	1.2	0.0	0.7
3390-1-R	1.5	0	0.1	4.5	0.3	3.3	72.6	10.1	4.6	1.2	1.1	0.1	0.7
3390-1-R*	1.2	0	0.1	4.7	0.3	1.9	59.9	17.1	12.6	0.6	1.3	0.1	0.4
3390-1-M	2.8	0	0.1	4.0	0.3	2.8	69.8	10.6	7.1	0.9	1.2	0.0	0.4
3390-1-M	2.0	0	0.1	4.9	0.4	3.3	70.3	11.1	4.9	1.2	1.2	0.1	0.7
3390-1-M	1.5	0	0.1	4.4	0.3	3.2	73.4	9.5	4.3	1.3	1.3	0.0	0.8
3390-1-M	1.5	0	0.1	4.5	0.3	2.8	72.7	10.0	5.1	1.1	1.3	0.0	0.7
3390-1-M	1.8	0	0.1	4.2	0.3	3.1	73.5	9.6	4.7	1.1	1.2	0.0	0.6
3390-1-M	1.5	0	0.1	4.7	0.3	2.9	71.6	10.7	5.1	1.1	1.2	0.1	0.7
3390-1-M	1.5	0	0.1	4.5	0.3	3.2	72.6	10.2	4.3	1.2	1.3	0.0	0.8
3390-1-M	1.8	0	0.1	4.4	0.3	2.9	72.0	10.4	5.2	1.1	1.2	0.1	0.6
3390-1-M	1.5	0	0.1	4.4	0.3	2.6	73.6	10.0	4.5	1.1	1.2	0.1	0.7
3390-1-M	2.3	0	0.1	4.3	0.3	3.0	73.0	9.7	4.5	1.1	1.2	0.0	0.6

SAMPLE	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
3390-8-R*	1.0	0	0.1	4.9	0.3	1.6	59.2	18.9	11.9	0.5	1.2	0.1	0.3
3390-8-R	2.1	0	0.1	4.2	0.3	2.7	71.9	10.2	5.6	1.0	1.2	0.1	0.6
3390-8-R	1.5	0	0.1	4.4	0.3	2.3	72.5	10.4	5.7	0.9	1.4	0.1	0.6
3390-8-R*	1.2	0	0.1	4.9	0.3	1.7	59.7	18.2	11.6	0.6	1.3	0.1	0.4
3390-8-R*	1.5	0	0.1	4.7	0.3	1.6	58.7	18.5	12.2	0.6	1.3	0.1	0.4
3390-8-R	1.8	0	0.1	4.2	0.3	2.9	73.4	9.2	5.2	1.1	1.3	0.0	0.6
3390-8-R*	1.1	0	0.1	4.7	0.3	1.5	56.9	19.3	14.1	0.5	1.1	0.1	0.2
3390-8-R	2.2	0	0.1	4.6	0.3	3.0	71.4	10.0	5.2	1.1	1.2	0.1	0.7
3390-8-R	1.7	0	0.1	4.6	0.4	2.4	72.5	11.0	4.8	0.9	1.3	0.1	0.5
3390-8-R	2.4	0	0.1	4.7	0.3	2.9	74.0	8.4	4.0	1.1	1.2	0.0	0.7
3390-8-R	1.9	0	0.1	4.6	0.4	3.0	72.7	9.7	4.8	1.0	1.2	0.0	0.6
3390-8-R	2.0	0	0.1	4.4	0.3	2.8	73.2	9.7	4.5	1.0	1.3	0.0	0.6
3390-8-R	1.5	0	0.1	4.3	0.3	2.6	71.8	10.7	5.8	1.0	1.3	0.1	0.6
3390-8-R	1.5	0	0.1	4.4	0.3	2.7	72.6	10.5	4.9	1.0	1.3	0.1	0.6
3390-8-R	2.0	0	0.1	4.9	0.4	3.3	71.1	10.4	4.9	1.1	1.1	0.1	0.6
3390-8-R	2.1	0	0.0	4.5	0.4	3.6	73.0	8.8	4.3	1.3	1.2	0.0	0.7
3390-8-R	2.2	0	0.1	5.1	0.4	2.9	67.6	12.3	6.5	1.1	1.2	0.1	0.7
3390-8-R	1.8	0	0.1	4.2	0.3	2.6	73.5	9.9	4.8	1.0	1.3	0.1	0.6
3390-8-R	1.7	0	0.1	4.7	0.3	3.0	72.5	9.9	4.6	1.2	1.3	0.1	0.7
3390-8-R	1.7	0	0.1	4.6	0.4	2.8	73.7	9.5	4.1	1.1	1.3	0.1	0.7
3390-8-R	1.5	0	0.1	4.5	0.3	3.0	74.7	8.5	4.2	1.2	1.2	0.0	0.7
3390-8-R	1.5	0	0.1	4.4	0.4	1.9	70.0	11.8	7.2	0.8	1.4	0.1	0.5
3390-8-R	1.7	0	0.1	4.4	0.3	2.5	71.8	11.1	5.2	1.0	1.3	0.1	0.6
3390-8-R	1.4	0	0.1	4.5	0.4	2.8	73.3	9.7	4.9	1.1	1.2	0.1	0.6
3390-8-R	1.5	0	0.1	4.8	0.4	3.0	72.6	10.6	4.1	1.1	1.2	0.1	0.7
3390-8-R*	1.4	0	0.1	5.8	0.4	2.9	54.0	20.0	13.0	0.8	1.1	0.1	0.7
3390-8-R	1.4	0	0.1	4.4	0.3	2.7	71.2	10.8	6.0	1.0	1.3	0.1	0.4
3390-8-R	1.7	0	0.1	4.6	0.4	2.8	72.6	10.0	5.1	1.0	1.2	0.1	0.6
3390-8-R*	1.0	0	0.1	4.6	0.3	1.6	59.6	18.5	12.3	0.5	1.2	0.1	0.3
3390-8-R*	1.1	0	0.1	4.6	0.3	1.4	56.5	20.4	13.4	0.5	1.3	0.1	0.3
3390-8-M	1.8	0	0.1	4.7	0.4	3.3	70.1	11.1	5.5	1.2	1.1	0.1	0.7
3390-8-M	1.5	0	0.1	4.3	0.3	3.0	73.0	10.3	4.3	1.1	1.2	0.1	0.7
3390-8-M	1.9	0	0.1	4.5	0.4	3.7	73.1	8.9	4.2	1.3	1.2	0.0	0.7
3390-8-M	1.6	0	0.1	4.4	0.3	2.5	73.4	9.7	5.1	1.0	1.3	0.1	0.7
3390-8-M	1.3	0	0.1	4.4	0.3	3.0	73.7	9.6	4.4	1.1	1.3	0.0	0.7
3390-8-M	2.1	0	0.1	4.3	0.3	3.2	74.0	8.9	4.1	1.2	1.2	0.1	0.6
3390-8-M	2.1	0	0.1	3.9	0.3	1.6	71.6	11.9	5.7	0.7	1.5	0.1	0.5
3390-8-M	1.6	0	0.1	4.6	0.3	2.8	71.0	11.8	4.8	1.0	1.3	0.1	0.6
3390-8-M	2.1	0	0.1	4.8	0.4	3.2	70.3	10.7	5.2	1.2	1.2	0.1	0.7
3390-8-M	1.6	0	0.1	4.5	0.3	2.9	72.7	9.9	4.8	1.1	1.3	0.0	0.7

The above data demonstrate a substantial increase in oleic acid (18:1) in seeds from each of the transgenic lines. The increase in oleic acid is at the expense of linoleic and linolenic acids, both of which were decreased in the transgenic lines. Increases in 18:0 and 20:0 fatty acids were also observed. Based on these data, the 5 null seeds present in the Random population can be identified, and are marked on Table 5 with an asterisk (\*). All of the seeds in the Maroon populations from each transgenic line have the observed altered fatty acid composition, confirming that the altered fatty acid composition is the result of expression of the *crtB* gene.

10 The trends in fatty acid composition data in the transgenic seeds which indicate positive and negative correlations of fatty acid composition changes with the observed increase in 18:1 levels are provided in Figures 5-7. The increase in 18:1 correlates with the decreases in 18:2 and 18:3. (Figure 5). The increase in 18:1 also correlates with an increase in both 18:0 and 20:0, but little effect on 16:0 was seen (Figure 6). The increase in 18:0 also correlated with an increase in 20:0 (Figure 7).

15 F. Carotenoid Analysis of Mature Seeds from *crtE* Transgenic Plants

Carotenoids were analyzed in mature T2 seeds of 3392 *B. napus* plants transformed to express the *E. uredovora crtE* gene. Approximately two fold increases in levels of lutein and  $\beta$ -carotene was observed in seeds of plant 3392-SP30021-16. Lycopene was also detected in these seeds and is undetectable in seeds of 20 untransformed control plants. Analysis of seeds from 7 additional 3392 transformants did not reveal significant increases in the carotenoid levels.

G. Analysis of Chlorophyll and Tocopherol Levels in *crtE* Transgenic Plants

Chlorophyll levels were analyzed using a spectrophotometric assay (Bruinsma, J. 1961, A comment on the spectrophotometric determination of chlorophyll, 25 Biochem Biophys Acta, 52:576-578) in mature T2 seeds of transgenic 3392 *B. napus* plants. Levels in 3392 transgenic plants were compared to seeds of transgenic *B. napus* plants expressing phytoene synthase ( *crtB* ) and to nontransformed control plants. Results are shown in Table 6 below.

TABLE 6

5

Pigment concentration (µg/gFW)

	Gene and sample	Total carotenoids	Total chlorophyll
<b>Phytoene synthase</b>			
10	27 DPA SP001 control	53	676
	27 DPA T4 3390-1-6	354	282
	40 DPA SP001 control	47	471
	40 DPA T4 3390-1-6	534	179
15	50 DPA SP001 control	16	125
	50 DPA T4 3390-1-6	648	125
<b>GGPP synthase</b>			
20	35 DPA SP3002I control	68	407
	35 DPA T2 3392-4	65	660
	35 DPA T2 3392-16	73	648
	Mature SP3002I control	21	35
	Mature T2 3392-4	25	31
	Mature T2 3392-16	50	60

25

Chlorophyll concentrations of the 35 DPA seeds of two lines were increased by approximately 60% compared to the levels of the control plant. The initial results demonstrate that the GGPP synthase gene increased the GGPP substrate availability for chlorophyll biosynthesis during seed development. Mature seeds of the 3392-16 line had higher chlorophyll and carotenoid concentrations than those of the control.

30

#### H. Carotenoid Analysis of Mature Seeds from *crtI* Transgenic Plants.

Carotenoids were analyzed in mature T2 seeds of 9010 *B. napus* plants transformed to express the antisense lycopene  $\epsilon$ -cyclase gene. Seeds of nine transgenic plants were analyzed for carotenoid content. An approximately two fold increase in levels of lutein,  $\beta$ -carotene and total carotenoids was observed in seeds of one line, 9010-SP3002I-10, when compared to control plants.

I. Carotenoid Analysis of Mature Seeds from *crtB + crtI* Transgenic Plants

Carotenoid levels of Mature 9009 T2 seeds were extracted and quantified on an HPLC as follows. Approximately 100mg of seeds were ground in a mortar and 5 pestle in 3ml extraction solvent ( hexane/acetone/ethanol ( 50/25/25 v/v) with 0.2ml of an internal standard ( 5mg/ml  $\beta$ -apo-8' carotenal ( dissolved in 100 $\mu$ l hexane), in acetonitrile/methylene chloride/methanol ( 50/40/10, v/v)). The extraction solution was transferred to a new glass tube, and the remaining seed was again extracted with the extraction solvent and pooled with first extraction solution. The extraction was 10 repeated until no color was visible in the extraction solution. Pooled extracts were mixed by vortexing briefly, then centrifuged for approximately 5 minutes. The resulting supernatant was transferred to a new tube and dried under nitrogen gas. The residue was resuspended in 2ml of hexane. Potassium hydroxide, in methanol, was added to a final concentration of 5%, and the solution was incubated overnight in the 15 dark at 4°C. Another 2ml of hexane was then added to the solution with 1ml of saturated sodium chloride. The solution was mixed briefly by vortexing and centrifuged for approximately 5 minutes. The upper hexane layer was removed and transferred to a new glass tube. The remaining bottom phase was again extracted with hexane and centrifuged. The upper phase was combined with the previous hexane 20 phase. This was repeated until the hexane phase was colorless. The pooled hexane phases were dried under nitrogen gas, and the residue was dissolved in 2.0ml of acetonitrile/methylene chloride/methanol ( 50/40/10 v/v). The solution was filtered through a 0.45 $\mu$ m filter and collected in a brown autosampler vial. Carotenoid 25 concentrations were determined on a Hewlett Packard 1050 High-Performance Liquid Chromatograph (HPLC), and isocratic separation of carotenoids was performed on a Hewlett Packard reverse phase C-18 ( 5 $\mu$ ) column (4.6 mm x 20cm) at 30°C. The mobile phase was acetonitrile/ methylene chloride/ methanol (80/10/10, v/v) with a flow rate of 1.0ml/min and a sample injection volume of 20 $\mu$ l (running time of 22min). Routine detection of colored carotenoids is at 450 nm, phytoene at 280 nm,

and phytofluene at 365 nm. Spectral scans for peak purity were made at 250 nm and 600 nm. Spectra of peaks at the upslope, apex, and downslope are normalized and overlaid. Superimposing spectra were taken as evidence of peak purity. The results are shown in Table 7 below. Carotenoid levels are presented as  $\mu\text{g/gFW}$ .

5

TABLE 7

10	Sample ID #	Lutein	Lycopene	$\alpha$ -Carotene	$\beta$ -Carotene	Phytoene	Total
	SP30021 control 3390-SP001-1-6-15	36	ND	ND	4	ND	40
15	(T5 Homo)	54	4	552	638	277	1525
	9009-SP30021-1	44	44	336	691	42	1157
	9009-SP30021-6	53	87	689	1118	152	2099
	9009-SP30021-9	48	34	487	798	194	1561
	9009-SP30021-10	33	25	248	489	34	829
20	9009-SP30021-12	31	ND	ND	2	ND	33
	9009-SP30021-14	42	37	404	791	81	1355
	9009-SP30021-15	37	15	137	278	ND	467
	9009-SP30021-16	50	38	428	828	65	1409

The results demonstrate that as with plants transformed to express *crtB* alone, 25 plants expressing *crtB* and *crtI* contain significant increases in total carotenoid levels. Furthermore, it is apparent that expression of *crtI* with *crtB*, leads to further 30 modification of the phytoene pools which accumulate in *crtB* transformants. Phytoene levels were reduced from about 20% of total carotenoids in lines transformed with *crtB* alone, to 4% to 7% of total carotenoids in the *crtB* + *crtI* lines. This indicates 35 that phytoene desaturase can have a synergistic effect with phytoene synthase in increasing the metabolic flux through the carotenoid/ isoprenoid pathway, and provides for even greater increases in a desired carotenoid compound, such as  $\alpha$ -carotene and  $\beta$ -carotene, than is obtained by expression of *crtB* alone. The increased flux also appears to result in increased total carotenoid production, in addition to the 40 composition shift from phytoene. For example, the carotenoid levels in the

segregating T2 seed populations of 9009-10 are significantly higher than those detected in the 3390 homozygous seed population in 3390-1-6-15.

5 J. Carotenoid Analysis of mature Seeds from *crtB* + Antisense  $\epsilon$ -Cyclase Transgenic Plants

Carotenoids from mature seeds from 9002 transformants were extracted and analyzed using the method described in example 2I above. These results are shown in Figure 14.

10 The initial results show a modification to the ratio of  $\beta$ -carotene to  $\alpha$ -carotene. In addition, several lines show a significant reduction in lutein levels when compared to nontransgenic controls. In 9002 T2 lines,  $\beta$ -carotene to  $\alpha$ -carotene ratios averaged 1.5, ranging from 1.1 to 2.5. For comparison, T2 3390 lines containing *crtB*, the ratio of  $\beta$ -carotene to  $\alpha$ -carotene averaged 1.9, ranging from 1.5 to 2.4.

15 K. Carotenoid Analysis of Mature Seeds from *crtB* Transgenic Cotton Plants

Mature 3390 T2 seeds from cotton were collected and carotenoid extracts were prepared and analyzed according to the method described in 2I above. These results are shown in Table 8 below. Carotenoid levels are presented as  $\mu\text{g/gFW}$ .

20

TABLE 8

Sample ID #	Lutein	Lycopene	$\alpha$ -Carotene	$\beta$ -Carotene	Phytoene	Total
C130 control	2	ND	ND	ND	ND	2
25 3390-C130-5-1	7	ND	486	420	517	

20 An approximately 3 fold increase in lutein was observed in seeds of plant 3390-C130-5-1. Alpha-carotene,  $\beta$ -carotene and phytoene were also observed in this line and are undetectable in nontransformed control plants. With  $\beta$ -carotenoid levels being 20 fold higher than those of  $\alpha$ -carotene. Total carotenoid levels were increased by more than 250 fold, with phytoene accounting for approximately 80% of that total.

L. Carotenoid Analysis of Mature Seed from *crtB + crtW* Transgenic Plants

Carotenoid levels of Mature 6204 T2 seeds were extracted and quantified on an HPLC as follows. Approximately 100mg of seeds were ground in a mortar and 5 pestle in 3ml extraction solvent ( hexane/acetone/ethanol ( 50/25/25 v/v) with 0.3ml of an internal standard ( 5mg/ml  $\beta$ -apo-8 $\beta$  carotenal ( dissolved in 100 $\mu$ l hexane), in acetonitrile/methylene chloride/methanol ( 50/40/10, v/v)). The extraction solution was transferred to a new glass tube, and the remaining seed was again extracted with the 2 ml extraction solvent and pooled with first extraction solution. The extraction 10 was repeated until no color was visible in the extraction solution. Pooled extracts were mixed by vortexing briefly, then centrifuged for approximately 5 minutes. The resulting supernatant was transferred to a new tube and dried under nitrogen gas. The dried sample was stored in the dark overnight at 4°C. The residue was resuspended in 3ml of hexane and 1 ml methanol, and 1 ml of saturated sodium chloride was added 15 and mixed. The samples were centrifuged briefly, and the upper phase was transferred to a new tube. The remaining bottom phase was again extracted with 2 ml hexane and centrifuged. The upper phase was combined with the previous hexane phase. This was repeated until the hexane phase was colorless. The pooled hexane phases were dried under nitrogen gas, and the residue was dissolved in 2.0ml of 20 acetonitrile/methylene chloride/methanol ( 50/40/10 v/v). The solution was filtered through a 0.45 $\mu$ m filter and collected in a brown autopsampler vial. Carotenoid concentrations were determined on a Hewlett Packard 1100 High-Performance Liquid Chromatograph (HPLC), and isocratic separation of carotenoids was performed on a Spherisorb ODS2 reverse phase C-18 ( 5 $\mu$ ) column (4.6 mm x 25cm) at 30°C. The 25 mobile phase was 82 acetonitrile/ 10 dioxane /8 methanol (v/v) containing 150 mM ammonium acetate/ 0.1 triethylamine, with a flow rate of 1.0ml/min and a sample injection volume of 20 $\mu$ l (running time of 46 min). Routine detection of colored carotenoids is at 450 nm, phytoene at 280 nm, and phytofluene at 365 nm. Spectral

scans for peak purity were made at 250 nm and 600 nm. Spectra of peaks at the upslope, apex, and downslope are normalized and overlaid. Superimposing spectra were taken as evidence of peak purity. The results are shown in Table 10 below, and an HPLC chromatogram is shown in Figure 17. Table 9 below describes the relevant 5 peak retention times shown in Figure 17. Carotenoid levels are presented as  $\mu\text{g/gFW}$ .

**Table 9.**

Ret Time [min]	Area [mAU*s]	Amt/Area	Amount [ $\mu\text{g/gFW}$ ]	Compound Name
3.500				Astaxanthin
5.428	721.34	$4.3 \times 10^{-3}$	59.33	Lutein
5.831	169.38	$4.26 \times 10^{-3}$	13.81	Zeaxanthin
6.533	527.83	$4.45 \times 10^{-3}$	44.88	Canthaxanthin
7.651	553.82	$3.59 \times 10^{-3}$	38.02	Internal Std
14.403				Echinone
18.453	68.21	$7.02 \times 10^{-3}$	9.16	Lycopene
22.278				Neurosporene
31.363	2966.38	$3.52 \times 10^{-3}$	199.36	$\alpha$ -carotene
33.870	2854.27	$3.86 \times 10^{-3}$	210.64	$\beta$ -carotene
44.166	524.14	$1.59 \times 10^{-2}$	158.86	Phytoene
<b>Totals:</b>			<b>734.05</b>	

**Table 10.** Carotenoid concentrations of canola seeds from selected T2 6204-SP30021 lines.

5	Sample ID	Segreg ratio	Carotenoid Concentration (µg/ g FWt.)							Total
			Lutein	Canth	Lycopene	a-Carotene	b-Carotene	Phytoene		
	SP30021	homo	21	ND	ND	ND	ND	ND	ND	21
	3390-SP30021-12	homo	44	ND	9	416	578	279	1326	
	6204-SP30021-1	3:1	41	22	9	223	252	192	744	
	6204-SP30021-2	15:1	43	24	9	231	283	236	831	
	6204-SP30021-3	3:1	51	8	6	165	268	65	568	
	6204-SP30021-5	63:1	56	25	11	292	296	305	992	
	6204-SP30021-6	no fit	61	47	9	206	218	165	720	
	6204-SP30021-7	3:1	41	13	8	180	232	160	634	
	6204-SP30021-8	3:1	41	16	6	68	108	54	299	
	6204-SP30021-9	>63:1	57	39	10	233	245	245	837	
	6204-SP30021-10	no fit	33	9	7	165	24	103	343	
	6204-SP30021-11	3:1	39	7	9	198	266	145	662	
	6204-SP30021-12	15:1	40	15	10	212	281	172	734	
	6204-SP30021-13	15:1	52	44	9	207	223	247	788	
	6204-SP30021-15	no fit	54	20	8	205	291	160	738	
	6204-SP30021-21	3:1	44	11	8	142	216	126	551	
	6204-SP30021-24	3:1	47	9	9	149	202	89	509	
	6204-SP30021-25	15:1	37	33	8	235	257	243	819	
	6204-SP30021-28	15:1	46	11	9	225	288	123	707	
	6204-SP30021-29	null	18	ND	ND	ND	ND	ND	18	
	6204-SP30021-30	3:1	33	11	8	203	246	154	659	
	6204-SP30021-36	15:1	31	17	7	197	275	95	628	
	6204-SP30021-37	3:1	38	10	7	137	181	117	490	
	6204-SP30021-41	3:1	42	9	8	250	339	170	821	
	6204-SP30021-42	3:1	32	4	6	178	216	103	539	
	6204-SP30021-43	15:1	54	25	10	242	304	151	792	
	6204-SP30021-44	no fit	48	27	7	226	249	129	692	

10 The initial results demonstrate that as with plants transformed to express *crtB* alone, plants expressing *crtB* and *crtW* contain significant increases in total carotenoid levels. Furthermore, the results show an increase in the levels of canthaxanthin, when compared to the levels obtained from seeds of plants transformed with *crtB* alone, as well as nontransformed control plants. In addition, other products were also produced in plants expressing *crtB* and *crtW*. Increased levels of

echineone, a reaction intermediate, as well as a putative 4-keto- $\alpha$ -carotene (Figure 17).

**M. Carotenoid Analysis of Mature Seeds from *crtB* Transgenic *Arabidopsis* Plants**

Mature 3390 T2 seeds from *Arabidopsis* were collected and carotenoid extracts were prepared and analyzed according to the method described in 2I above. These results are shown in Table 11 below. Carotenoid levels are presented as  $\mu\text{g/gFW}$ .

10 **Table 11. Carotenoids of T2 *Arabidopsis* seeds transformed with *crtB*.**

Sample ID	Date of Harvest	Carotenoid concentration ( $\mu\text{g/g FWt.}$ )					Total
		Lutein	Lycopene	$\alpha$ -Carotene	$\beta$ -Carotene	Phytoene	
AT001-50 VAR	6/4/98	18	ND	ND	2	ND	20
3390-AT001-1	6/4/98	24	ND	7	20	7	58
3390-AT001-2	6/17/98	57	5	68	139	98	368

15 Initial results indicate that seeds from one line of *Arabidopsis* transformed with napin-*crtB* had an 18-fold increase in total carotenoid concentration. This line also demonstrate an approximately 70 fold increase in  $\beta$ -carotene levels (Table 11).

20 **Example 3 Crosses of *crtB* Plants**

A. Transgenic Oil Traits

25 To evaluate the high oleic trait of the napin-*crtB* transgenic plants in conjunction with expression of other oils traits, crosses off 3390-1-6-8 with a mangosteen thioesterase (5266) and a nutmeg thioesterase (3854; see WO 96/23892) were made. Crosses were also made with two low linoleic (LPOO4 and LP30108) varieties. Half-seed analyses of carotenoids and fatty acid composition were conducted on the segregating seeds, and the average of the half seed values are shown 30 below in Tables 12 and 13.

TABLE 12  
Carotenoid Levels in Half Seeds Resulting from 3390 Crosses

	Cross	Lutein	Lycopene	$\alpha$ -Carotene	$\beta$ -Carotene	Total
5	F1 3390-SP001-1-6-8 x SP30021	21.6	26.2	271.5	413.1	732.4
	F1 3390-SP001-1-6-8 x 5266-SP30021-5-26	18.0	21.7	187.9	284.1	511.7
	F1 3390-SP001-1-6-8 x 5266-SP30021-35-2	16.2	22.1	223.0	318.4	579.7
	F1 3390-SP001-1-6-8 x 5266-SP30021-35-12	19.5	22.9	196.8	312.8	552.0
10	F1 3390-SP001-1-6-8 x LP30108-19		23.7	22.7	213.4	355.0
	F1 LP30108-19 x F1 3390-SP001-1-6-8	16.4	19.6	156.7	224.5	417.2

15	STRAIN_ID	%14:0 %16:0 %18:0 %18:1 %18:2 %18:3 %20:0						
		(3390-SP001-1-6-8 X SP30021)	0.05	3.55	1.70	74.78	11.29	5.71
	(3390-SP001-1-6-8 X 5266-SP30021-35-12)	0.06	3.84	11.37	62.86	11.06	5.08	3.38
	(3390-SP001-1-6-8 X 5266-SP30021-35-2)	0.06	3.68	11.27	64.80	9.81	5.16	3.04
	3390-SP001-1-6-8 X 5266-SP30021-5-26	0.06	3.66	15.36	60.78	9.30	4.77	3.87
	(3390-SP001-1-6-1 X 3854-SP30021-20-3)	2.69	9.80	3.65	64.62	9.72	4.57	1.51
	(3390-SP001-1-6-1 X 3854-SP30021-20-1)	6.14	16.35	5.12	54.91	8.23	4.23	2.03
	(3390-SP001-1-6-1 X 5266-LP004-2-31)	0.07	3.82	11.67	64.52	11.46	3.14	3.08
	(3390-SP001-1-6-8 X LP30108-19)	0.05	3.80	1.44	73.66	14.02	3.93	0.67
	(LP30108-19 X 3390-SP001-1-6-8)	0.04	3.31	1.79	79.69	9.26	2.97	0.75
	SPO01-4-10	0.07	4.44	0.99	56.06	21.79	14.31	0.44
	3390-SPO01-1-6-8	0.04	3.46	1.44	77.26	9.30	5.71	0.63

As the above results demonstrate, a dramatic increase (100 to 200 fold) in  $\alpha$ - and  $\beta$ -carotene as well as a 60 fold increase in total carotenoids may be obtained by transformation of plants for expression of an early carotenoid biosynthesis gene under the regulatory control of promoter preferentially expressed in plant seed tissue. This 5 increase in flux primes the pathway for the production of speciality products as described above, and also results in increased production of  $\alpha$ -tocopherol (Vitamin E).

Furthermore, it is evident that the fatty acid composition can also be altered in the transgenic plant seeds. In this manner, seeds can be used to produce novel 10 products, to provide for production of particular carotenoids, to provide high oleic oils, and the like.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference 15 to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the 20 appended claims.

## IN THE CLAIMS

What is claimed is:

1. A method for altering xanthophyll content in seed of a host plant, said method comprising the steps of
  - transforming cells of a host plant with a construct comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from a carotenoid biosynthesis gene coding region, and a transcriptional termination region,
  - producing a transformed host plant from said transformed cells, and
  - growing said transformed host plant or progeny thereof containing said construct under conditions whereby seed is produced having an altered xanthophyll content.
2. The method of Claim 1 whereby a novel xanthophyll is produced in said seed to effect said alteration.
3. The method of Claim 1 whereby the level of at least one xanthophyll produced in said seed is increased to effect said alteration.
4. The method of Claim 1 whereby the level of at least one xanthophyll produced in said seed is decreased to effect said alteration.
5. The method of Claim 1, wherein said DNA sequence reduces the expression of a carotenoid biosynthesis gene native to said host plant by antisense or cosuppression.
6. The method of Claim 5, wherein said carotenoid biosynthesis gene is lycopene  $\epsilon$ -cyclase.
7. The method of Claim 1, whereby said alteration of xanthophyll content is effected by the action of a protein expressed from said carotenoid biosynthesis gene DNA sequence on at least one carotenoid substrate present in said plant seed.
8. The method of Claim 7, wherein said carotenoid substrate is selected from the group consisting of  $\alpha$ -carotene,  $\beta$ -carotene,  $\gamma$ -carotene,  $\delta$ -carotene, zeaxanthin, canthaxanthin, echinenone, hydroxycanthaxanthin,  $\beta$ -cryptoxanthin, adonixanthin,  $\alpha$ -cryptoxanthin, and astaxanthin.
9. The method of Claim 1, wherein said carotenoid biosynthesis gene is selected from the group consisting of phytoene synthase, phytoene desaturase,  $\beta$ -carotene hydroxylase, lycopene  $\beta$ -cyclase, and  $\beta$ -carotene ketolase.

10. The method of Claim 1, wherein said carotenoid biosynthesis gene is not native to said host plant.
11. The method of Claim 1, wherein said carotenoid biosynthesis gene is from a prokaryote.
12. The method of Claim 1, wherein said host plant is an oilseed *Brassica* plant.
13. The method of Claim 1, wherein said host plant is cotton.
14. The method of Claim 1, wherein said transcriptional initiation region is from a gene preferentially expressed in *Brassica* seed tissue.
15. The method of Claim 14, wherein said transcriptional initiation region is from a napin gene.
16. A transformed host plant having altered xanthophyll content in seed and produced according to the method of Claim 1.
17. Seed of a transformed host plant according to Claim 15.
18. A method for producing an increased level of xanthophyll in a seed from a host plant, said method comprising transforming said host plant with 1) an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from a first carotenoid biosynthesis gene coding region, and a transcriptional termination region, and 2) an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from a second carotenoid biosynthesis gene coding region, and a transcriptional termination region, and wherein said first and second genes are carotenoid biosynthesis genes selected from the group consisting of phytoene synthase,  $\beta$ -carotene hydroxylase, and  $\beta$ -carotene ketolase.
19. The method of Claim 18, wherein said first and second carotenoid biosynthesis genes encode  $\beta$ -carotene hydroxylase and  $\beta$ -carotene ketolase.
20. The method of either Claim 19, wherein astaxanthin content in said seed are increased.
21. The method according to Claim 18 further comprising transforming said host plant with 3) an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a

plastid transit peptide, a DNA sequence derived from a third carotenoid biosynthesis gene coding region, and a transcriptional termination region, wherein said third carotenoid biosynthesis gene encodes a carotenoid biosynthesis gene selected from the group consisting of phytoene synthase,  $\beta$ -carotene hydroxylase, and  $\beta$ -carotene ketolase.

22. The method of Claim 18, wherein said first and second carotenoid biosynthesis genes encode phytoene synthase and  $\beta$ -carotene hydroxylase.
23. The method of Claim 21, wherein said first and second carotenoid biosynthesis genes encode phytoene synthase and  $\beta$ -carotene hydroxylase and said third carotenoid biosynthesis gene encodes phytoene desaturase.
24. The method of Claim 19, wherein zeaxanthin content in said seed are increased.
25. The method of Claim 18, wherein said first and second carotenoid biosynthesis gene encodes phytoene synthase and  $\beta$ -carotene ketolase.
26. The method of Claim 21, wherein said first and second carotenoid biosynthesis genes encode phytoene synthase and  $\beta$ -carotene ketolase and said third carotenoid biosynthesis gene encodes phytoene desaturase.
27. The method of Claim 25, wherein canthaxanthin content in said seed are increased.
28. The method of Claim 26, wherein canthaxanthin content in said seed are increased.
29. The method of Claim 25, wherein echinenone content in said seed are increased.
30. The method of Claim 26, wherein echinenone content in said seed are increased.
31. The method according to Claim 21 further comprising transforming said host plant with 4) an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from a fourth carotenoid biosynthesis gene coding region, and a transcriptional termination region, wherein said fourth carotenoid biosynthesis gene encodes phytoene desaturase, phytoene synthase,  $\beta$ -carotene hydroxylase and  $\beta$ -carotene ketolase.
32. The method of Claim 31, wherein astaxanthin content in said seed are increased.

33. The method according to Claim 21 wherein transcription from said third DNA sequence results in inhibition of transcription of an endogenous plant gene encoding lycopene  $\epsilon$ -cyclase.

34. The transformed seed of any of one of Claim 1, Claim 16, Claim 21, Claim 31, or Claim 33, wherein said seed produces increased content of at least one xanthophyll compound of interest, said compound of interest selected from the group consisting of zeaxanthin, canthaxanthin, echinenone, hydroxycanthaxanthin,  $\beta$ -cryptoxanthin, adonixanthin,  $\alpha$ -cryptoxanthin, and astaxanthin.

35. The method of Claim 1, wherein said alteration of xanthophyll content is effected by the action of the protein expressed from said carotenoid biosynthesis gene DNA sequence on a compound on a carotenoid substrate present in said plant seed.

BgIII

AGATCTGCTA GAGACCTTTC CTTTCAAC AGAACTGAGA 30  
 AGATCTGCTA GAGACCTTTC CTTTCAAC AGAACTGAGA 30

CTCTTCGGCT GTGACACAG TCAGGCCGTGC CTCTTACGGG CAATCGCCG CAGTGGCTCC 120

ATTCGGGGC CTCATAATCCA TGTACTGGATT CCCAGTGAAG AAGGTAAACA CTGACATTAC 180

TTTCATTAAAG AGCAATTGCTG GAAGAGTAAAG GTGCATGAAAT ATTCGGTGT TACTCAATCA 240

TGCTTCTGAA ACCATGGCAG TTGGCTCGAA AAGTTTTCGG ACAGCCCTCAA AGTTTATGAA 300

TGCAAAACC CGGGCGAGGG TACTGTATGCT CTACGCCCTGG TGCCGGCATT GTGACCGATGT 360

TATGACCAT CAGACCCCTAG SCTTTCAAGGC CGGGAGGCT GCCTTACAAA CGCCCGAACAA 420

ACGTCTGATG CAACTTGAAG TGAAACGGG CCAGGCCTAT GCAGGGATCGC AGATGGACGA 480

ACGGCCGTTT CGGGCTTTTC AGGAAAGTGGC TATGGCTCAT GATATGGCC CGGCTTACGC 540

GTTTGATCAT CTGGAGGCT TCGCCATGGA TGTACGGAA GCGCAATAACA GCCAAACTGGA 600

TCATACGGCTG CGCTTATGCT ATCACGTTGC AGGCGTTGTC GGCTTGTATGA TGGCGCAAAT 660

FIGURE 1  
1/2

CATGGCGTG CGGGATAACG CCACGCTGGA CCGGCCCTGT GACCTGGGC TGGCATTTCA 720  
GTTGACCAT ATTCTCGCG ATATTCTGGA CGATGCCAT GCGGCCGCT GTTATCTGCC 780  
GGCAGAGCTGG CTGAGGCGATG AAGGTCTGAA CAAAGAGAAT TATGCCGCAC CTGAAAAACCG 840  
TCAGGGCGCTG AGCCGATTCG CCCTGCGTTT GTTGAGGGAA GCAGAACCTT ACTATTTTC 900  
TGCACACAGGC CGCCTGGCAG GGTGCCCCCT GCGTTCCGCC TGGGCAATCG CTACGGGAA 960  
GCAGGGTTAC CGGAAATAATG GTGTCAAAAGT TGAAACAGGCC GGTCAAGAAG CCTGGGATCA 1020  
GGGGCAAGTCA ACGACCAAGGC CCCGAAATAAT AACGCTGCTG CTGGCCGCCT CTGGTCAGGC 1080  
CCTTACTTCG CGGATGCCGC CTCATCCCTCC CCGCCCTGCC CATCTCTGGC AGGCCCGCT 1140  
CTAGGGCCAT GTCTTCCCG GAGGCTCCGA ATTATCGATG AATTGGAGCT CGGTACCCGG 1200  
BamHI  
|  
GGATCCCTTA GAGTGACCT GCAGGGCATGC AA 1232

FIGURE 1  
2/2

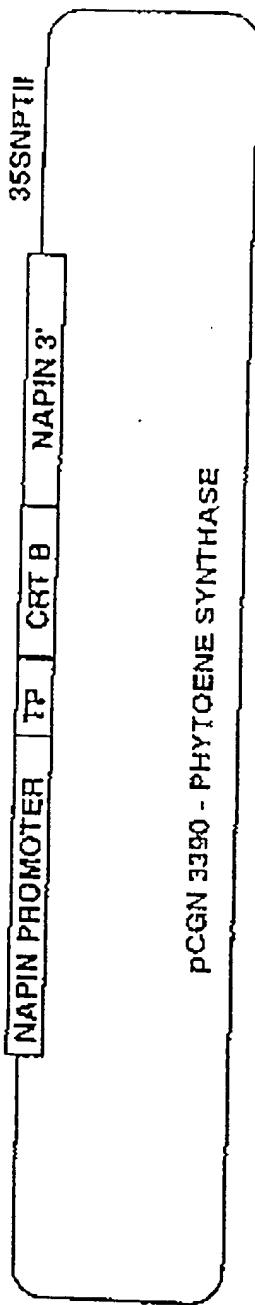


FIGURE 2A

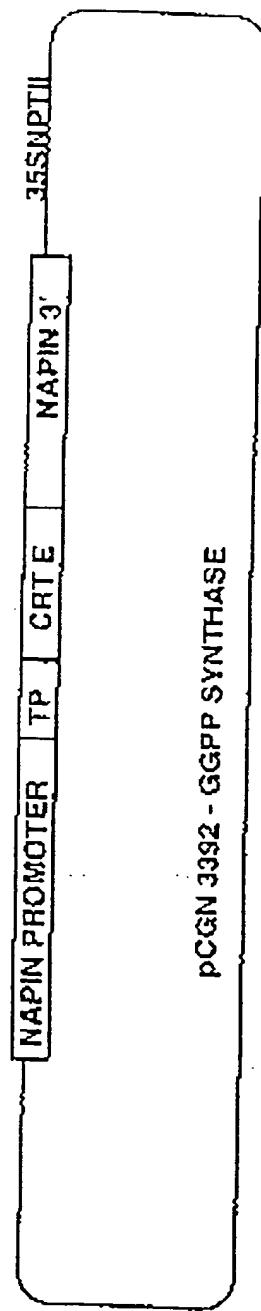


FIGURE 2B

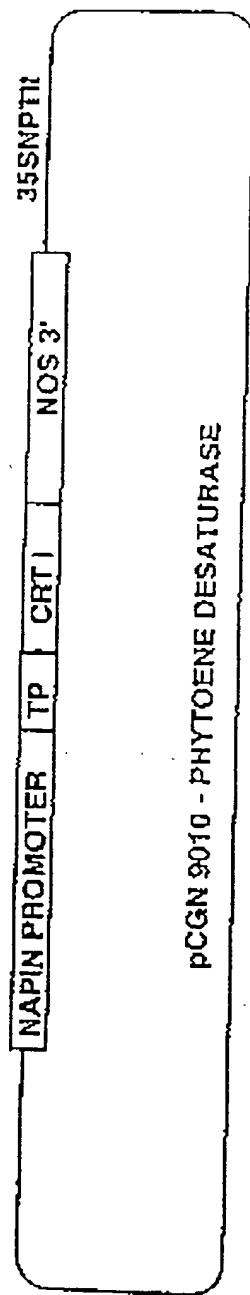


FIGURE 2C

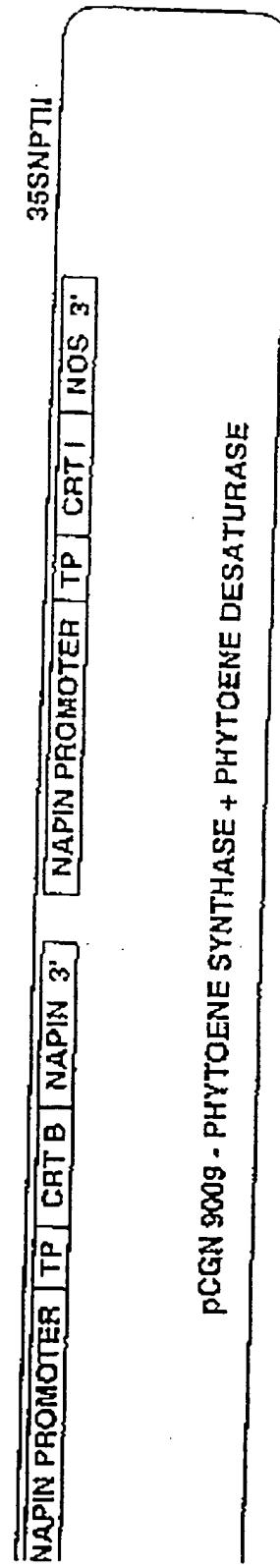


FIGURE 2D

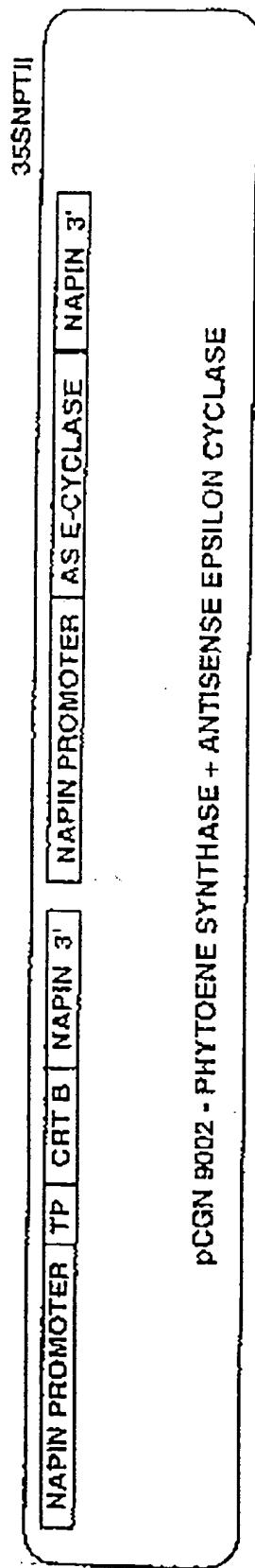


FIGURE 2E

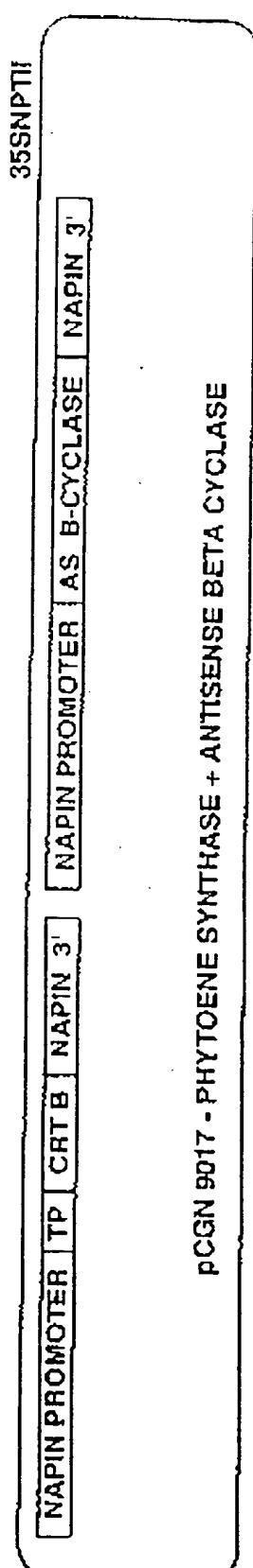
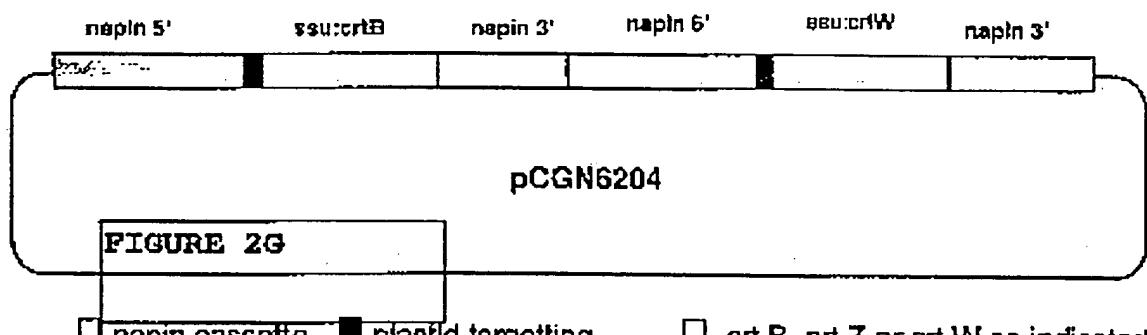
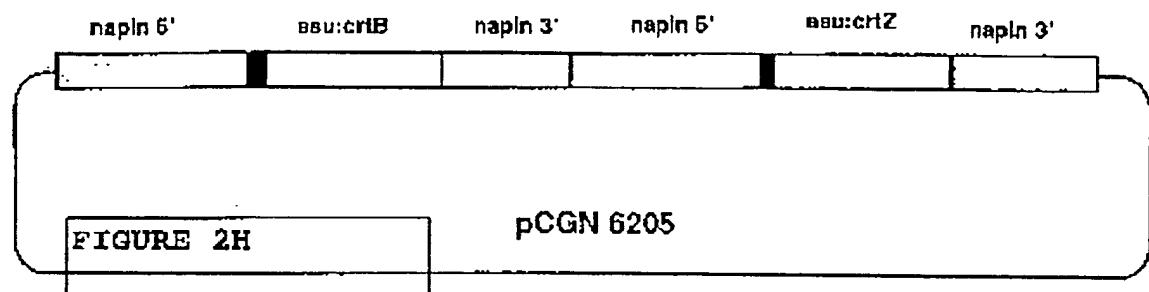
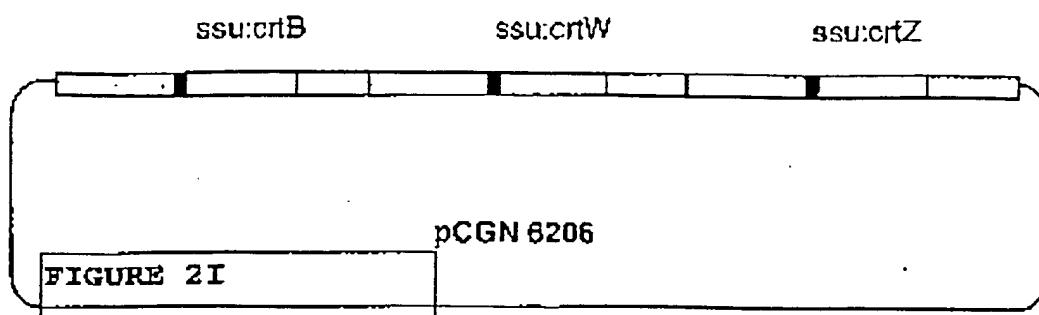


FIGURE 2F



napin cassette    plastid targetting    crt B, crt Z or crt W as indicated

CONTROL SAP

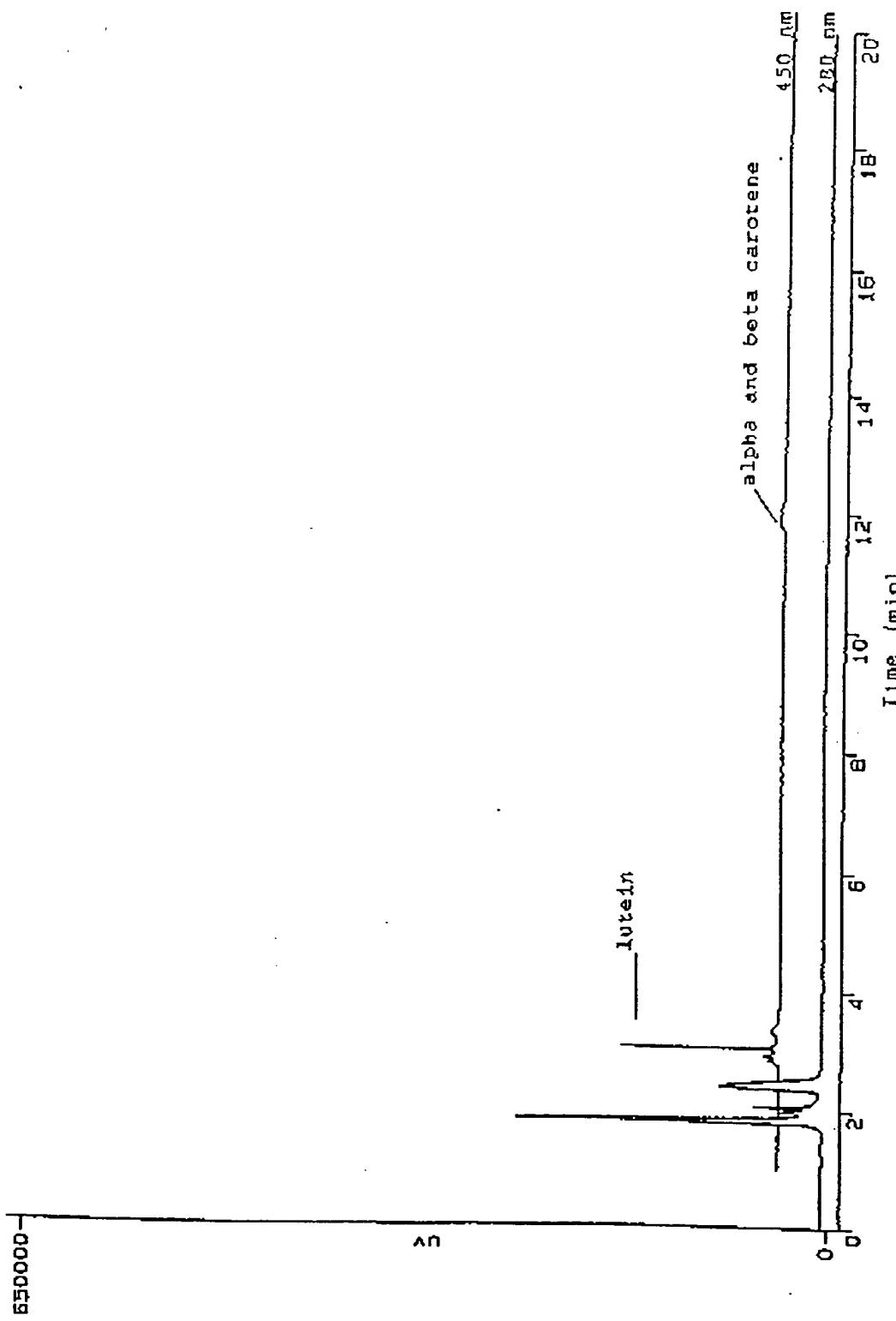


FIGURE 3 1/1

3390 SAP

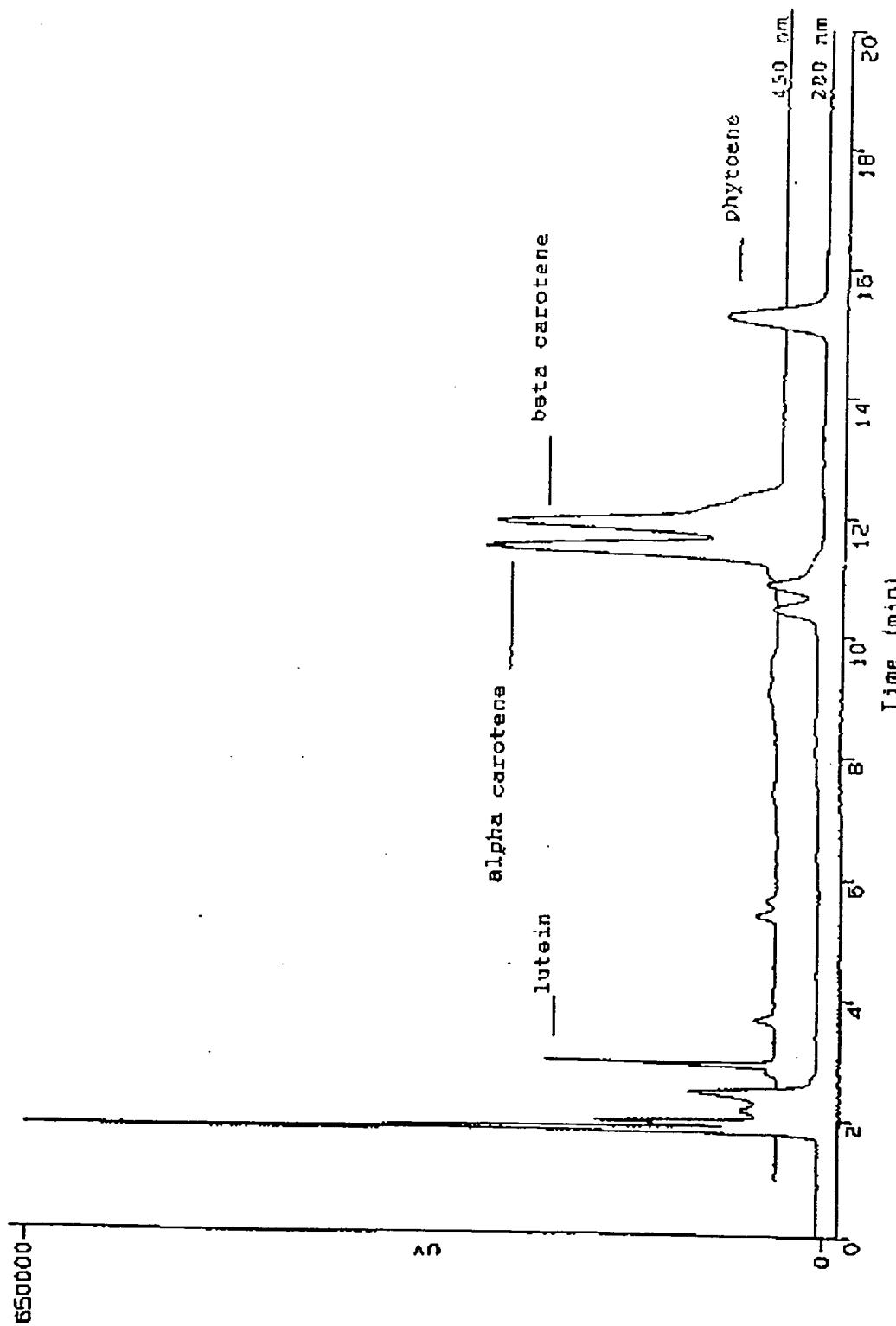


FIGURE 4 FIGURE 4 1/1

## 18:1 vs 18:2 and 18:3 in 3390s

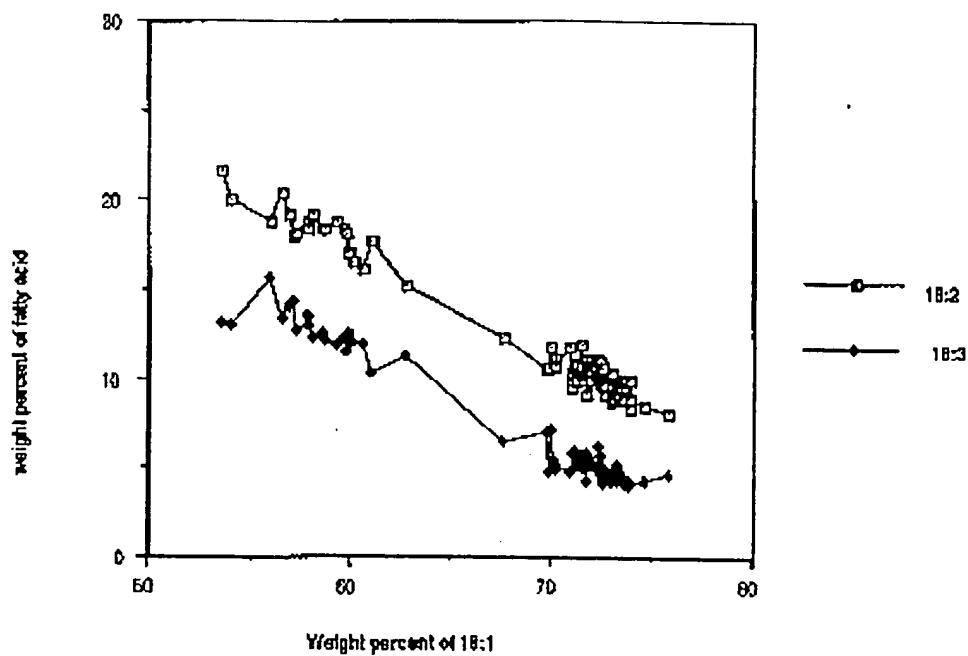


FIGURE 5

1/1

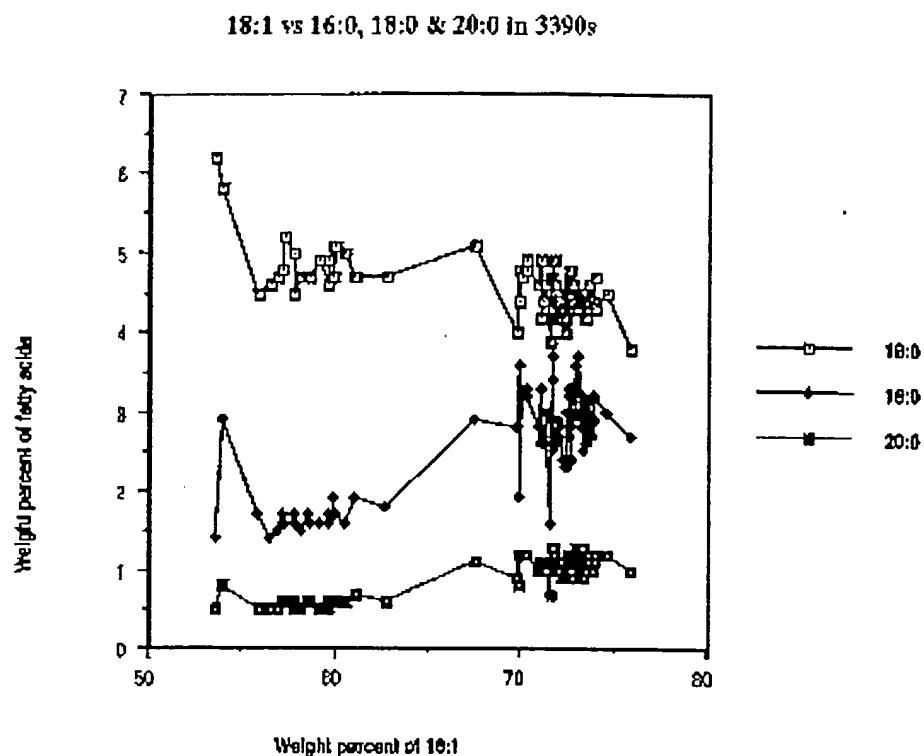


FIGURE 6

1/1

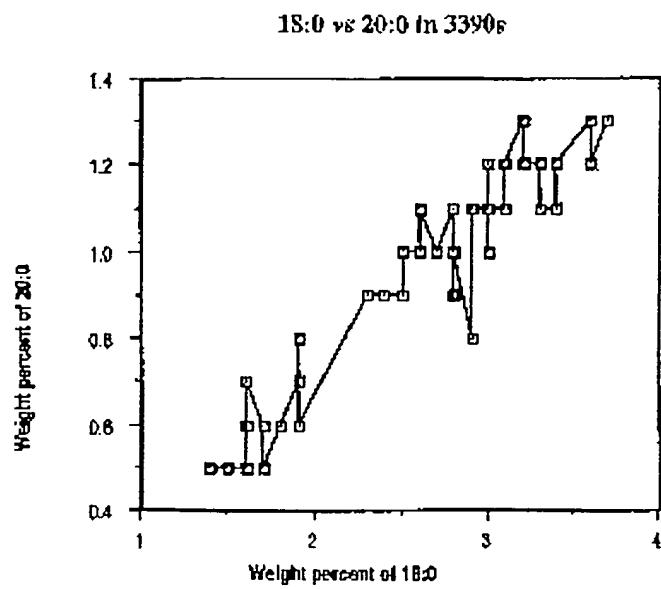


FIGURE 7

1/1

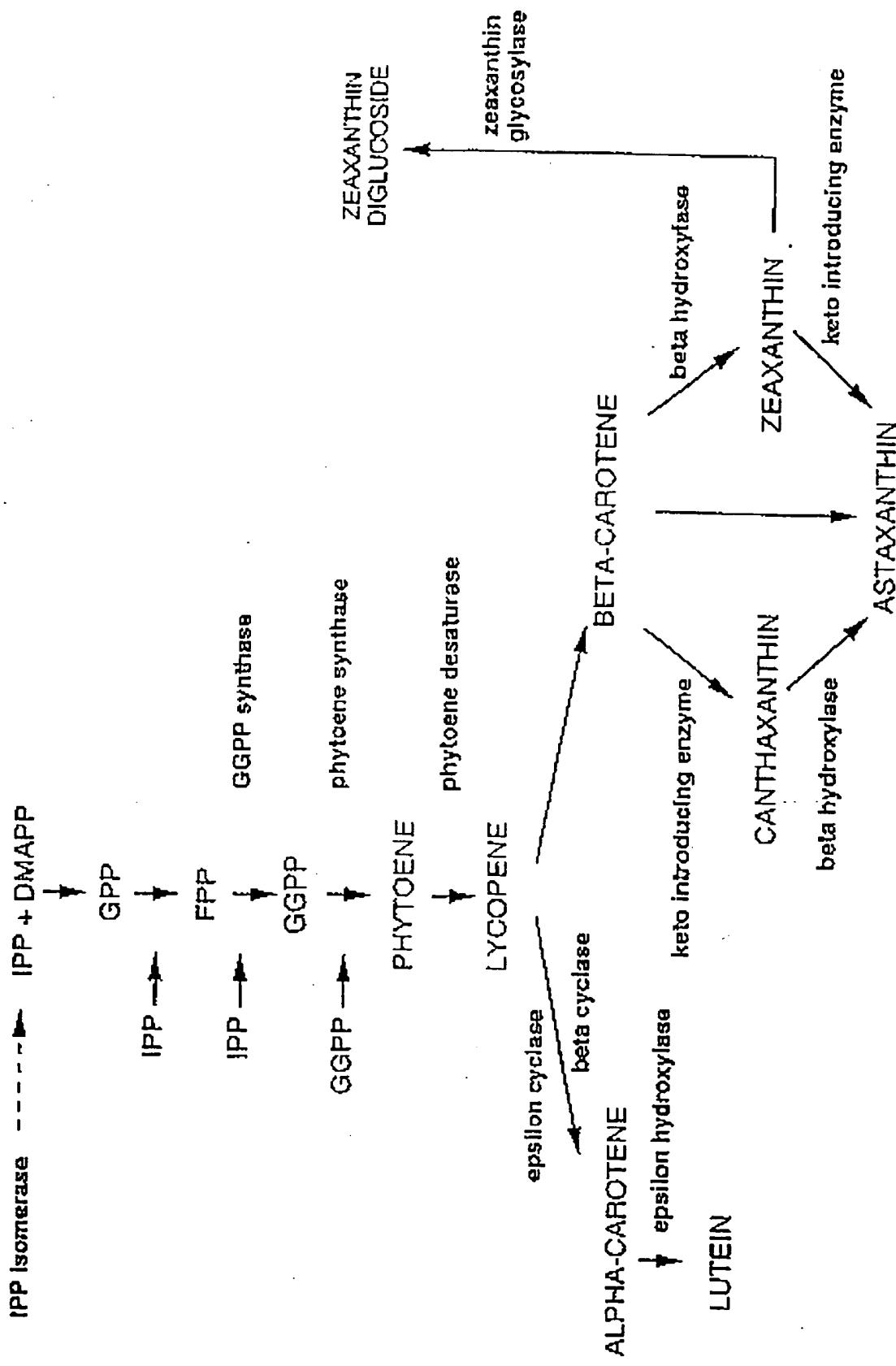


FIGURE 8

FIGURE 9  
1/2

>AluI            >AluI                            >HaeIII  
 |                    |                                    |  
 |                    500                                    |  
 \*                    \*                                    \*  
 CTTCTGGAGCAGCTCTGGGAGCTCTTGCATATCCAGTGGAGGGCTAGACTCTG  
  
 >HinfI           >Sau3AI  
 |                    |                                    |  
 560                    580                                    600  
 |                    |                                    \*  
 TCCAAACTCTTACGGCTTGGAGGTGGGGGGAAAGAGTCCATATGATCCAGAGCAGA  
  
 >AluI  
 |                    |                                    |  
 |                    >MspI                                    |  
 |                    |                                    |  
 620                    640                                    660  
 \*                    \*                                    \*  
 TGGTGTTCAGGATTACAGAGATTATAACAAACAGAAATCGGAGCTTAGAAGCTGAAT  
  
 >BinfI  
 |                    |                                    |  
 680                    700                                    720  
 |                    |                                    \*  
 ATCCAACTGTCTCTACGGCATGCCATATGAAAGAGCAGACTTCTTGAGGAGACAT  
  
 >AluI  
 |                    |                                    |  
 740                    760                                    780  
 \*                    \*                                    \*  
 GTCTTGCCTAAAGATGTATGCCCCCTTGGATTTCCTTAAAGAGCTCTTGTTGAGAT  
  
 >BinfI  
 |                    |                                    |  
 |                    800                                    |  
 \*                    \*                                    |  
 820                    840                                    \*  
 TAGAGACACTCGGAATCCGAATACTAAAGACTTACGAGAGGAATGGCTTATATCCAG  
  
 >AluI  
 |                    |                                    |  
 |                    >HinfI                                    |  
 |                    |                                    |  
 860                    880                                    900  
 \*                    \*                                    \*  
 TAGGTCTTCCCTGECAAACACGGAAACAAAGATTCGCCTTGGCGCTGCTAGCA  
  
 >SpcI           >BamHI  
 |                    |                                    |  
 >EcoR2           >BstXI                            >HaeIII                            |  
 |                    |                                    |                                    |  
 920                    940                                    960  
 \*                    \*                                    \*  
 TGGTACATCCCGAACAGAAGCGAAATCCAGCAGACTGGCGCCGTTACTAGTGGATCC  
 GA

FIGURE 9

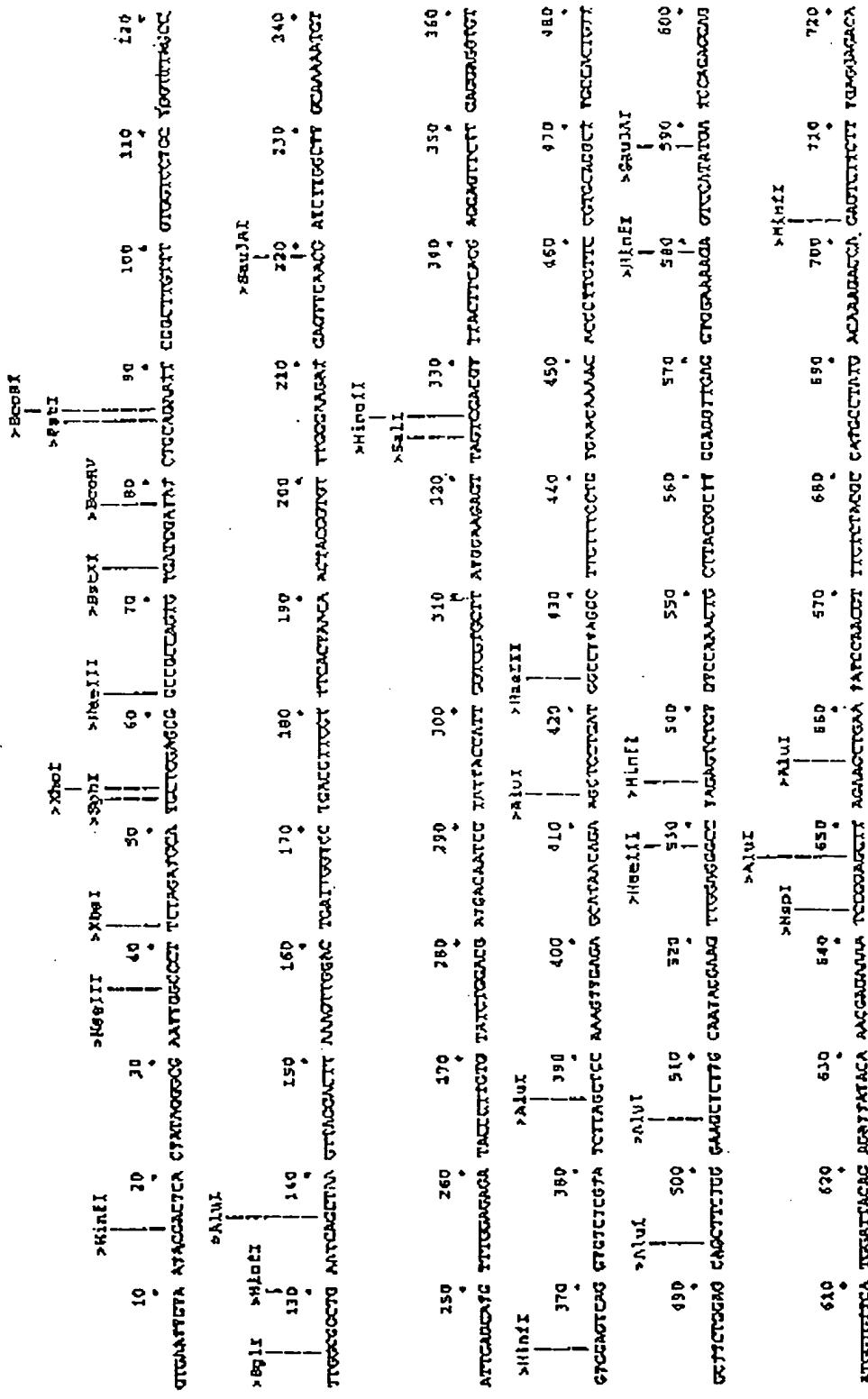


FIGURE 10  
1/2

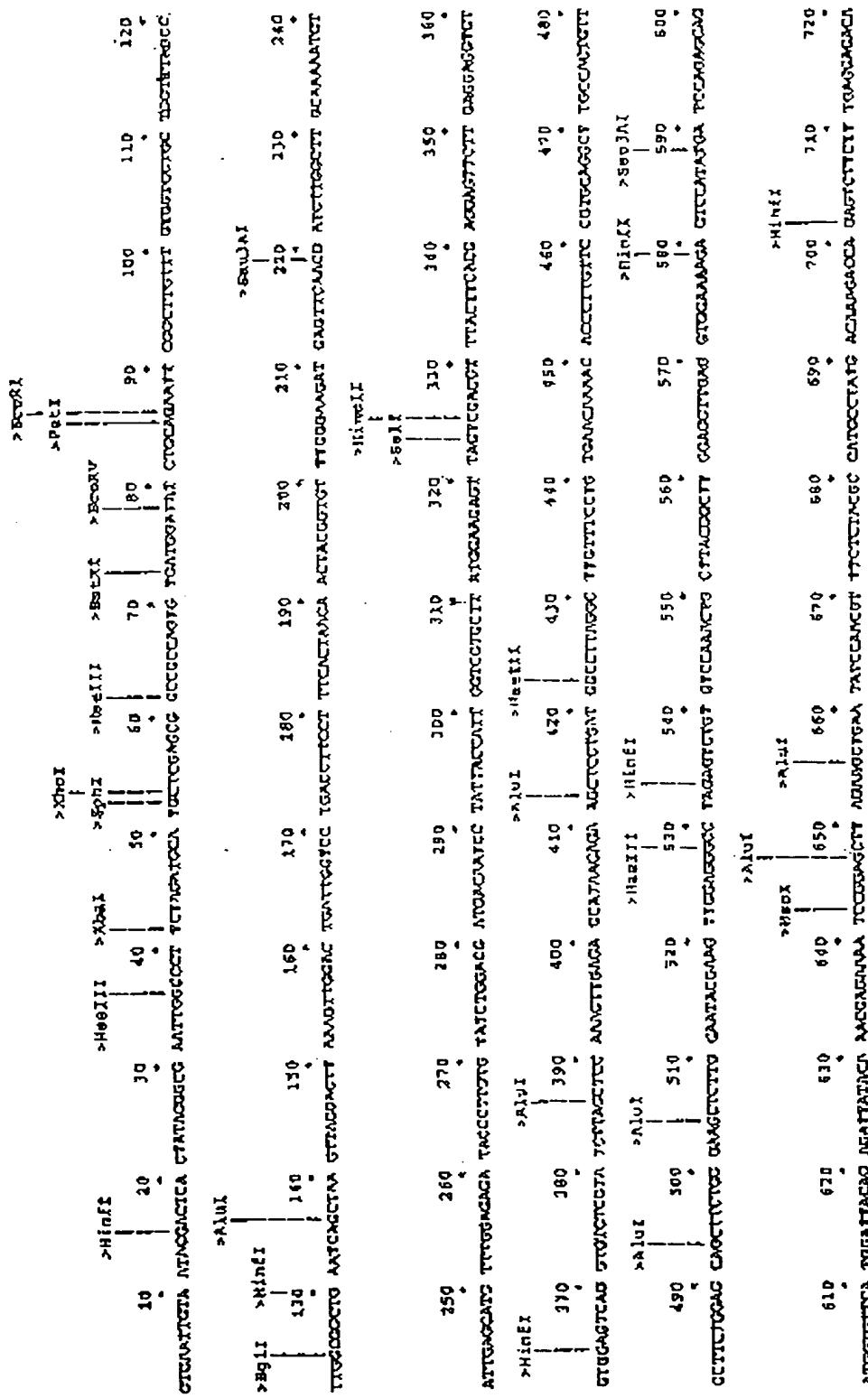


FIGURE 10  
1/3

60	*	GAGCTCGGAT CCACCTAA CGGCCGCG AG TGTGCTGGAA TTCCGGCTTCT ATCTTGTCAC
120	*	AAATTGTTGA TCATCTTGGC AAGGAAACA GTTCCCTTCG TCATGATCTC CAACCTCGAG
180	*	GTATTAGAAG CATGCCAGA GAGGACAGC CCGAGGAAACA CCGGGTCCCCG GAGAACACGC
240	+	CTCGACGACA AGAACCTATG CCAGTAAACGC GTTCCAGGT CAAGAACGCC ATCAAAGAAC
300	*	CTCCTAGTAG CATCCAAATC AAGCTTCAGC AAAATAATCCA TCCCAAAAACA GAGGAACCTCC
360	*	CTCTGTCTCC CCCTCTCAAT AGGCACAAAG TCTCTCCACAA CCTCAGCCGA GAGCTCATCT
420	*	CTCTCTAAAGC CCTCTGTCTT ACCACCA ACCACCA AGGTACCGCA CTATAGCTT TGCAACTATC
480	*	GGAGGAGCTG CAGACTCCT AGCAACCATG TAACCAAGTCG AAGGATGAAAC CATCCCCGGCC

FIGURE 11-14

540  
GTACCGCCAA TGCCAAACAC TCTTGGGC AAGACCGTA AAGGACCTCC CATAAGGATC  
\*  
600  
ACACAAACGCT CGTCTTCCTC AATCGCTTC ACGTGTATCC CCAAATGTTT CAGCCTCGCA  
\*  
660  
ACCATCCTCT CTGGATATC TCCATCTTC AGACCCGGCC TGGCACAAAG AGACGTCCT  
\*  
720  
TCAAGAAAGA TCCCTTCTGA AGAAACGGC ATCGCGTACA GGAAACGTAGG GATCTTGCTG  
\*  
780  
TTCCCGCTCTT TAACCTCTGG GTACCGGTCA AGATGCCATT CTCTCCAGTC CATGAAACACC  
\*  
840  
ATCTTATCCA CATCAACGG GTGACCATCG ACCCTAGCAA TGATACCAAA AGCTTACTTGA  
\*  
900  
TACCCAGGT TATAGGGCTT ATCATTACTGA ACCAAGGATC TTGAAAAACC AGTAGGGTCTG  
\*  
960  
AGAAACAAAG AGGCTGAAAT CTTCACACCC TCACTGCAGA CAAACGTGA GTTAACCTCC

FIGURE 11  
24

1020 \*  
 TCGTCAACCA CGTCAGTGAC TTTAGCCTGA TGGAAATCTAA CACCCCTTGT GATGCCATTC  
 1080 \*  
 TGAAGCATCT TGGATTGAG CTTGTTACGG TTCACTCTTC CGTAAAGGCCG GGACAGGTCC  
 1140 \*  
 TTTTCGAGGC CGTCGTTCT GTAGGACGAGC GCGGCCGGACC AGCTGGCTTC GAGGGAGTCT  
 1200 \*  
 AGCAGTCCA TGGCTTCGAA CTCGTCAAACC CAAPCTCCGT AGTTTGTAGG CCAATGAGT  
 1260 \*  
 TTGGGGAG GATCGATGGA GCAGACAGAG AGTCCACCTT CGCAGACTTG CTGAGCCACG  
 1320 \*  
 GCTAAACCG CGGGGCCGCC GCCAACCGATA GCTAGATCAA CAACTTGTG CAGGGAAAGTG  
 1380 \*  
 TCGTTAAAG GAAAGGTCCAA GTCGGAGATT TCCTTCTTGG TTTCAGGAAC AAGATCCAAA  
 1440 \*  
 AGAGCACTAC TAGCACTAGT GATACTACTA CGGATTCTGA TTGCTCTTTT CTTCAAAACCA

FIGURE 11  
3/4

1500 \*  
AGCTTACCC TCGAAGGATT TCGACTTAT CTCTCGAACCC CAGGAACCTC AGGGATGAAA  
1560 \*  
AACTCGGACT TTGTTGGGTGT TTTCACAGA GTATCCATCG AATTCTGGCAG ATATCCATCA  
CACTGGCGGC CGCTCGGAGCA TGCATCTAGA

FIGURE 11  
4/4

Sample ID #	Segregation ratio	Carotenoid concentration ( $\mu\text{g/g FFW}$ )				Total
		Lutein	Lycopene	$\alpha$ -Carotene	$\beta$ -Carotene	
SP30021 control 1		24.4	ND	ND	1.9	26.3
SP30021 control 2		34.0	ND	ND	4.9	38.9
T2 3390-SP30021-1	3:1	33.5	6.1	229.0	385.7	654.3
T2 3390-SP30021-2	15:1	50.4	6.2	372.4	721.4	1150.4
T2 3390-SP30021-3	no fit	45.8	3.9	352.9	580.9	983.5
T2 3390-SP30021-4	3:1	31.0	4.9	306.1	463.3	805.3
T2 3390-SP30021-5	3:1	36.8	10.5	370.6	659.4	1077.3*
T2 3390-SP30021-6	15:1	46.9	9.1	445.1	797.0	1298.1
T2 3390-SP30021-7	15:1	51.2	7.4	494.9	941.4	1494.9
T2 3390-SP30021-8	no fit	41.9	11.3	468.4	904.3	1425.9
T2 3390-SP30021-9	>63:1	68.4	11.9	394.2	949.2	1423.7
T2 3390-SP30021-10	null	51.6	ND	12.6	22.8	87.0
T2 3390-SP30021-11	3:1	52.2	9.5	409.8	714.5	1186.0*
T2 3390-SP30021-12*	3:1	48.0	10.2	400.0	738.8	1197.0*
T2 3390-SP30021-13	3:1	66.1	3.9	98.1	216.0	384.1
T2 3390-SP30021-14	3:1	49.1	8.9	320.0	611.6	989.6
T2 3390-SP30021-15	null	27.0	ND	ND	1.2	28.2
T2 3390-SP30021-16	3:1	55.6	6.4	283.1	527.4	872.5
T2 3390-SP30021-17	3:1	53.0	9.1	324.9	614.3	1001.3
T2 3390-SP30021-18	>63:1	49.6	8.1	449.0	759.3	1256.0
T2 3390-SP30021-19	3:1	62.2	7.6	346.1	613.2	1029.1
T2 3390-SP30021-20	3:1	52.1	6.3	285.0	544.9	838.3
T2 3390-SP30021-21	3:1	56.2	4.1	187.9	334.2	582.4
T2 3390-SP30021-22	null	43.1	ND	ND	4.9	48.0
T2 3390-SP30021-23	3:1	71.0	10.9	358.6	693.9	1134.4*
T2 3390-SP30021-24	no fit	53.9	7.3	272.1	520.4	853.7
T2 3390-SP30021-25	3:1	31.9	12.2	309.1	580.9	934.1
T2 3390-SP30021-26*	3:1	34.3	9.3	311.2	584.4	939.2*
T2 3390-SP30021-27	3:1	52.6	9.8	299.8	686.3	1048.5*

FIGURE 12  
1/2

T2 3390-SP30021-28	no fit	68.4	10.0	446.3	907.7	1432.4
T2 3390-SP30021-29	>63.1	85.1	8.5	459.4	822.9	1375.9
T2 3390-SP30021-30	3:1	63.7	5.8	356.9	598.4	1024.8
T2 3390-SP30021-31	3:1	76.0	7.3	302.5	527.1	912.9
T2 3390-SP30021-32	null	51.8	2.3	31.4	55.0	140.5
T2 3390-SP30021-33	3:1	36.3	6.9	283.1	546.9	875.2
T2 3390-SP30021-34	>63.1	86.9	12.1	502.3	808.3	1409.6
T2 3390-SP30021-35	3:1	39.3	8.1	224.5	461.0	732.9
T2 3390-SP30021-36	15:1	55.5	11.0	538.5	829.9	1434.9
T2 3390-SP30021-37*	3:1	50.3	10.0	291.1	625.9	977.3*
T2 3390-SP30021-38	3:1	70.5	8.1	309.0	576.1	963.7
T2 3390-SP30021-39	null	37.3	ND	ND	3.6	40.9
T2 3390-SP30021-40	3:1	37.5	1.8	251.1	505.2	796.0
T2 3390-SP30021-41	3:1	47.5	8.4	414.1	719.3	1189.3*
T2 3390-SP30021-42	3:1	42.6	5.1	230.3	352.9	630.9
T2 3390-SP30021-43	no fit	83.3	5.6	128.4	219.8	437.9
T2 3390-SP30021-46	3:1	21.6	1.4	211.2	368.3	602.5
T2 3390-SP30021-47	3:1	79.1	3.7	312.5	570.5	965.8
T2 3390-SP30021-48	3:1	45.3	3.0	225.2	401.5	675.0
T2 3390-SP30021-49	15:1	28.3	1.6	346.0	677.2	1053.1
T4 3390-SP001-1-6-13	Hom	52.4	1.5	439.5	669.3	1162.7

FIGURE 12  
2/2

Sample ID #	Segregation status	Carotenoid concentration (µg/gFW)					Total
		Lutein	Lycopene	α-Carotene	β-Carotene	Total	
T3 3390-SP001-4-12	Homo	43.9	17.2	282.1	636.8	980.0	
T3 3390-SP001-5-7	Het	50.7	6.3	190.6	386.8	634.4	
T3 3390-SP001-5-12	Homo	45.5	19.5	255.9	633.4	954.3	
T3 3390-SP001-11-5	Homo	46.5	12.8	372.2	538.4	969.9	
T3 3390-SP001-11-9	Homo	54.0	10.2	406.0	556.0	1026.2	
T3 3390-SP001-14-2	Homo	59.7	12.5	342.4	764.0	1178.6	
T3 3390-SP001-14-6	Homo	66.3	12.9	431.0	673.9	1184.1	
T3 3390-SP001-15-9	Homo	30.8	14.3	271.8	559.8	876.7	
T3 3390-SP001-15-12	Homo	39.6	13.1	241.7	649.1	943.5	
T3 3390-SP001-16-3	Homo	49.9	17.1	230.2	519.7	816.9	
T3 3390-SP001-16-6	Homo	35.5	21.1	263.8	547.7	868.1	
T3 3390-SP001-35-2	Het	37.6	7.2	125.4	313.9	484.1	
T3 3390-SP001-35-10	Homo	43.7	16.6	234.7	503.9	798.9	
T3 3390-SP001-35-12	Homo	50.2	21.3	361.7	695.7	1128.9	
T3 3390-SP001-8-3	Het	41.4	9.9	178.2	434.4	663.9	
T3 3390-SP001-8-9	Homo	39.1	18.2	309.3	505.0	871.6	
T3 3390-SP001-8-11	Homo	35.9	19.6	260.7	580.4	896.6	
T3 3390-SP001-18-3	Het	29.2	12.2	112.1	247.6	441.1	
T3 3390-SP001-16-10	Het	38.0	14.6	248.2	486.3	787.1	
T4 3390-SP001-1-6-1	Homo	27.8	20.5	248.7	379.1	676.1	
T4 3390-SP001-1-6-8	Homo	38.5	16.8	304.1	383.9	743.3	
VAR SP001-4-5		54.2	ND	ND	5.8	60.0	
VAR SP001-4-6		51.2	ND	ND	7.0	58.2	
VAR SP001-4-10		30.2	ND	ND	ND	30.2	

FIGURE 13  
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Sample ID #	Segregation ratio	Carotenoid concentration ( $\mu\text{g/gFW}$ )					
		Lutein	Lycopene	$\alpha$ -Carotene	$\beta$ -Carotene	Phytoene	Total
SP30021 control	None	21	ND	ND	2	ND	23
9002-SP30021-1*	3:1	20	2	394	618	210	1244
9002-SP30021-2	3:1	17	2	285	537	128	969
9002-SP30021-3	>64:1	19	7	489	689	381	1585
9002-SP30021-4	3:1	58	5	105	266	94	528
9002-SP30021-5	15:1	24	3	416	649	265	1357
9002-SP30021-6	3:1	13	2	324	546	176	1061
9002-SP30021-7	3:1	13	4	344	465	212	1038
9002-SP30021-8	15:1	12	3	449	690	224	1378
9002-SP30021-9	>64:1	24	5	499	724	313	1565
9002-SP30021-10	15:1	52	25	387	505	245	1214
9002-SP30021-11	3:1	29	2	301	480	187	999
9002-SP30021-12	>64:1	43	10	575	779	436	1843
9002-SP30021-13	3:1	19	3	357	509	279	1167
9002-SP30021-14	null	33	ND	ND	3	ND	36
9002-SP30021-15*	3:1	29	7	472	599	354	1461
9002-SP30021-16	64:1	40	3	315	436	203	997
9002-SP30021-17	15:1	25	7	322	467	144	967
9002-SP30021-18	>64:1	8	4	447	647	313	1419
9002-SP30021-19	15:1	38	17	537	570	327	1489
9002-SP30021-20*	3:1	32	8	363	629	173	1205
9002-SP30021-21	>64:1	1	6	468	736	348	1559
9002-SP30021-22	15:1	68	29	308	423	173	1001
9002-SP30021-23	15:1	51	20	449	553	423	1496
9002-SP30021-24	3:1	47	20	339	515	311	1232
9002-SP30021-25	null	27	ND	ND	2	ND	29
9002-SP30021-26*	3:1	4	3	346	605	150	1108
9002-SP30021-27	>64:1	25	5	416	698	376	1320
9002-SP30021-28	15:1	75	9	464	527	333	1408
9002-SP30021-29	null	32	ND	16	34	ND	82
9002-SP30021-30	3:1	25	9	316	525	182	1057
9002-SP30021-31	null	28	ND	ND	2	ND	30
9002-SP30021-32	3:1	29	5	198	283	132	647
9002-SP30021-33	15:1	30	40	408	557	324	1379
9002-SP30021-34	15:1	43	5	216	289	132	685
9002-SP30021-35	3:1	29	8	303	511	281	1132
9002-SP30021-36	3:1	26	9	324	402	157	918
9002-SP30021-37	3:1	34	11	263	418	143	869
9002-SP30021-39	15:1	54	13	219	420	118	824
9002-SP30021-40	15:1	30	7	382	716	235	1370
9002-SP30021-41	3:1	52	15	440	506	396	1409
9002-SP30021-42	3:1	49	20	317	516	170	1072
9002-SP30021-44	>64:1	34	7	368	647	310	1366
9002-SP30021-45	>64:1	45	9	429	636	402	1521
9002-SP30021-46	3:1	100	14	456	699	347	1617
9002-SP30021-48	3:1	37	5	191	354	231	818
9002-SP30021-50	64:1	51	22	522	736	303	1654

FIGURE 14

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10	20	30	40	
ATG GCT TCT ATG ATA TCC TCT GCT GTG ACA ACA GTC AGC CGT				
TAC CGA AGA TAC TAT AGG AGA AGG CGA CAC TGT TGT CAG TCG GCA				
M A S W I S S A V T T V S R>				
50	60	70	80	90
GCC TCT AGG GCG CHA TCC GCC GCA GTG GCT CCA TTC GGC GGC CTC				
CGG AGA TCC CCC GTT AGG CGG CGT CAC CGA GGT AAG CCG CCG GAG				
A S R G Q S A A V A P P G G I>				
100	110	120	130	
AAA TCC ATG ACT GGA TTC CCA GTG AAG GTC AAC ACT GAC ATT				
TTT AGG TAC TGA CCT AAG GGT CAC TTC TTC CAG TTG TGA CTG TAA				
K S W T G F P V K K V N T D I>				
140	150	160	170	180
ACT TCC ATT ACA AGC AAT GGT GGA AGA GTA AAG TGC ATG ACC AAC				
TGA AGG TAA TGT TCG TTA CCA CCT TCT CAT TTC ACC TAC TTG TTG				
T S I T S N G G R V K C M T N>				
190	200	210	220	
TTC TTG ATC GTT GTC GCT ACC GTT TTG GTT ATG GAG TTG ACT CCT				
AAG AAC TAC CAA CGA CGG CGA TGG CAA AAC CAA TAC CTC AAC TCA CGA				
F L - V V A T V L V M E L T A>				
230	240	250	260	270
TAC TCC GTC CAC AGA TGC ATC ATG CAC GGT CCA TTG CGT TGG GGT				
ATG AGG CAG GTG TCT ACC TAG TAC GGT CCA AAC CCA ACC CCA				
Y S V H R M I M H G P L G W G>				

FIGURE 15  
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280	280	290	290	300	300	310
TGG	CAC	AAG	TCC	CAC	GAG	GAG
ACC	GTC	TTC	AGG	GTC	GTC	GAC
W	H	K	S	H	E	G
320	320	330	330	340	350	360
AAC	GAC	TTC	TAC	GTT	ATC	GTC
TTC	CTG	AAC	ATG	TTC	GCT	TTG
N	D	L	Y	G	A	A
370	370	380	380	390	400	
TTC	ACC	GGT	TGG	ATC	TGG	
AAG	TGG	CAA	CCA	ACC	GCT	
P	T	V	G	W	W	
410	420	420	430	440	450	
TTC	GGT	ATG	ACT	GTC	TAC	
AAC	CCA	TAC	TGA	CAG	ATG	
L	G	M	T	V	Y	
460	470	470	480	490		
GGT	TTC	CAC	CAA	AGA	TAC	
CCA	AAC	CAG	GTC	GTC	ATC	
G	N	V	H	Q	CCA	
510	510	520	530	540		
GGT	TAC	GCT	AGA	AGA	TTC	
CCA	ATG	CGA	TCT	TCT	ATG	
G	Y	A	R	R	L	

FIGURE 15  
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550	560	570	580
GTC GAG GGT AGA GAC CAC TGT GTT TCT TTC GGT TTC ATC TAC GCT			
CAG CTC CCA TCT CTG GTG ACA CAA AGA AAG CCA AAG TAG TAG ATG CGA			
V E G R D H C V S F G I Y A>			
590	600	610	620
CCA CCA GTT GAT AAG TTG AAG CAA GAC TTG AAG ATG TCC GGT GTC			
GGT GGT CAA CTA TTC AAC TTC GTT CTG AAC TTC TAC AGG CCA CAG			
P P V D K L K Q D L K M S G V>			
640	650		
TTG AGA CCT GAG CCT CAA GAG AGA ACC TAG			
AAC TCT CGA CTC CGA GTT CTC TCT TGG			
L R A E A Q E R T>			

FIGURE 15  
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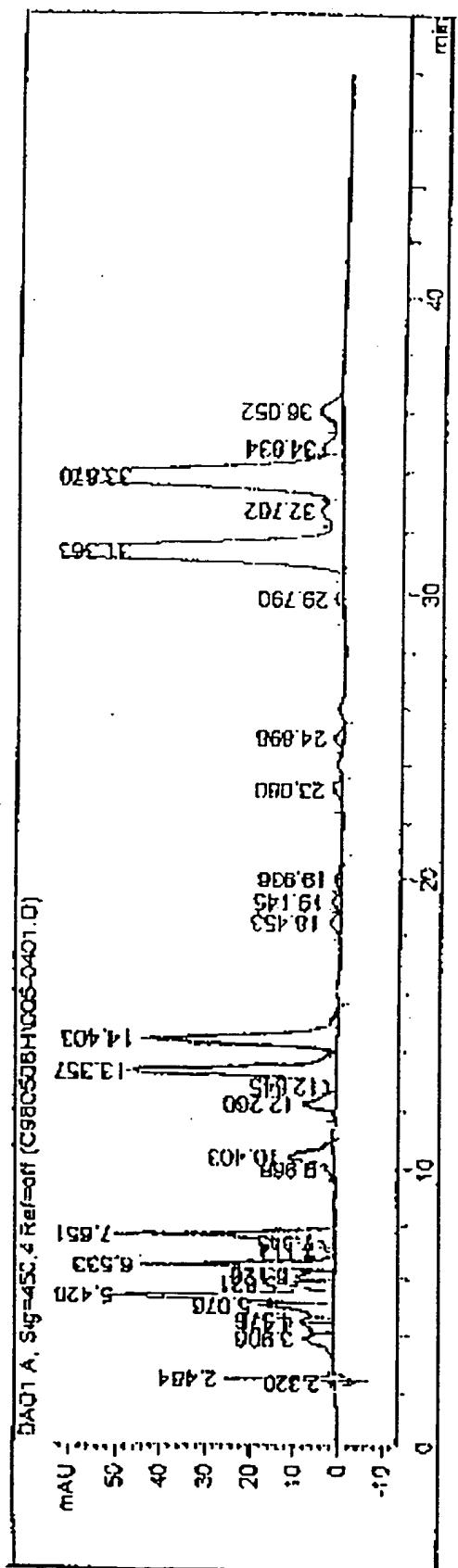
ATG	GCT	TCT	ATG	ATA	TCC	TCT	TCC	GCT	GTC	ACA	ACA	GTC	AGC	CGT	
TAC	CGA	AGA	TAC	TAT	AGG	AGA	AGG	CGA	CAC	TGT	TGT	CAG	TCG	GCA	
M	A	S	M	I	S	S	S	A	V	T	T	V	S	R>	
50	GCC	TCT	AGG	GGG	CAA	TCC	GCC	GCA	GTC	GCT	GTC	GCC	GGC	GGC	90
CGG	AGA	TCC	CCC	GTT	AGG	CGG	CGG	CGA	GGT	AAG	CCG	CCG	GAG	GAG	
A	S	R	G	Q	S	A	A	V	A	P	F	G	G	L>	
100	AAA	TCC	ATG	ACT	GGG	TTC	CCA	GTC	AAG	GTC	AAC	ACT	GAC	ATT	130
TTT	AGG	TCG	TAC	TGA	CCT	AAG	GCT	CAC	TTC	TTC	CAG	TTC	TTC	TAA	
K	S	M	T	G	F	P	V	K	K	V	N	T	D	I>	
140	ACT	TCC	ATT	ACA	AGC	AAT	GGT	CGA	AGA	GTC	AAG	TTC	ATG	TCC	180
TGA	AGG	TAA	TGT	TCG	TAA	CCA	CCT	TCT	CAT	TTC	ACG	TAC	AGG	CGA	
T	S	I	T	S	N	G	G	R	V	K	C	M	S	A>	
190	CAC	GCT	TTG	CCA	AAG	GCT	GAC	TTG	ACT	GCT	ACC	TCC	TTG	ATC	220
GTG	CGA	ACG	GGT	TTC	CGA	CTG	ACG	TGA	CGA	TGG	AGG	ACG	TTG	CAG	
H	A	L	P	K	A	D	L	T	A	T	S	L	I	V>	
230	TCC	GGT	GGT	ATC	GCT	GCT	TTG	TTG	GCT	TTC	TCC	TTG	ATC	GTC	270
AGG	CCA	CCA	CCA	TAG	TAG	CGA	CGA	CGA	ACC	AAC	CGA	AAC	GTG	CAC	
S	G	G	G	I	I	A	A	N	L	A	L	H	V	H>	
280	TTC	TGG	TTC	GAC	GCT	GCT	GCT	CAC	CCA	ATC	TTG	GCT	ATC	GCT	310
AAC	ACC	ACC	ACC	AGG	AAC	CTG	CGA	CGA	CGA	GGT	GGT	TAG	AAC	CGA	
L	W	F	L	D	A	A	A	H	P	I	L	A	I	A>	

FIGURE 16  
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320	330	340	350	360
ABC TTC TTG GGT TTG AAC TGG TTG TCT GTC GGT TTG TTC AAC ATC				
TTC AAG AAC CCA AAC TTG ACC AAC AGA CAG CCA AAC AAG TAG TAG				
N F L G L N W L S V G L P I I>				
370	380	390	400	
GCT CAC GAC GCT ATG CAC GGT TCC GTT GTC CCA GGT AGA CCA AGA				
CGA GTG CTG CGA TAC GTG CCA AGG CAA CAG GGT CCA TCT GGT TCT				
A H D A M H G S V V P G R P R>				
410	420	430	440	450
GCT AAC GCT GCT ATG GGT CAA TTG GTT TTG TGG TAC GCT GGT				
CGA TTG CGA CGA TAC CCA GTT ATC CAA AAC ACC AAC ATG CGA CCA				
A N A A M G Q L V L W L Y A G>				
460	470	480	490	
TTC TCT TTG AGA AAG ATG ATC GTT AAG CAC ATG GCT CAC CAC AGA				
AAG AGA ACC TCT TTC TAC TAG CAA TTC GTG TAC CGA GTG GTG TCT				
F S W R K M I V K H M A H H R>				
500	510	520	530	540
CAC GCT GGT ACT GAT GAC GAC CCA GAT TTC GAC CAC GGT GGT CCA				
GTG CGA CCA CGA TGA CTA CTG CTG GGT CTA AAG CTG GTG CCA CCA GGT				
H A G T D D P D F D H G G P>				
550	560	570	580	
GTT AGA TGG TAC GCT AGA TTC ATC GGT ACT TAC TTC GGT TGG AGA				
CAA TCT ACC ATG CGA TCT AAG TAG CCA TGA ATG AAG CCA ACC TCT				
V R W Y A R P I G T Y F G W R>				
590	600	610	620	630
GAG GGT TTG TTG TTG CCA GTC ATC GTT ACC GTT TAC GCT TTG ATC				
CTC CCA AAC AAC AAC GGT CAG TAG CAA TGG CAA ATG CGA AAC TAG				
E G L L P V I V T V Y A L I>				

TTC GGT GAC AGA TGG ATG TAC GTC GTC	640	TTC GGT GAC AGA TGG ATG TAC GTC GTC	650	TTC GGT GAC AGA TGG ATG TAC GTC GTC	660	TTC GGT GAC AGA TGG ATG TAC GTC GTC	670
AAC CCA CTG TCT ACC TAC ATG CAA CAG AAG ACC GGT AAC GGT AGG		L G D R W N Y V P W P L P S>		L G D R W N Y V P W P L P S>		L G D R W N Y V P W P L P S>	
ATC TTG GCT TCT ATC CAA TTG TTC GTT TTC	680	ATC TTG GCT TCT ATC CAA TTG TTC GTT TTC	690	ATC TTG GCT TCT ATC CAA TTG TTC GTT TTC	700	ATC TTG GCT TCT ATC CAA TTG TTC GTT TTC	710
TAG AAC CGA AGA TAG GTT AAC AAG CAA AAG CCA TGG ACC AAC GGT		I L A S I Q L P V F G T N L P>		I L A S I Q L P V F G T N L P>		I L A S I Q L P V F G T N L P>	
CAC AGA CCA GGT CAC GAC GCT TTC CCA GAC AGA CAC AAC GCT CGA	730	CAC AGA CCA GGT CAC GAC GCT TTC CCA GAC AGA CAC AAC GCT CGA	740	CAC AGA CCA GGT CAC GAC GCT TTC CCA GAC AGA CAC AAC GCT CGA	750	CAC AGA CCA GGT CAC GAC GCT TTC CCA GAC AGA CAC AAC GCT CGA	760
GTT G GTT CCA GTG CTG CGA AAG GGT CTG TCT GTG TTG CGA GCT		H R P G H D A P P D R H N A R>		H R P G H D A P P D R H N A R>		H R P G H D A P P D R H N A R>	
TCC TCC AGA ATC TCT GAT CCA GTC GTC	770	TCC TCC AGA ATC TCT GAT CCA GTC GTC	780	TCC TCC AGA ATC TCT GAT CCA GTC GTC	790	TCC TCC AGA ATC TCT GAT CCA GTC GTC	800
AGG AGG TCT TAG AGA CTA GGT CAA AGG AAC AAC TGG ACA AAG GTC		S S R I S D V V S L L T C F H>		S S R I S D V V S L L T C F H>		S S R I S D V V S L L T C F H>	
TTC GGT GCT TAC CAC CAC GAG CAC CAC TTG CAC CCA ACT GTC CCA	820	TTC GGT GCT TAC CAC CAC GAG CAC CAC TTG CAC CCA ACT GTC CCA	830	TTC GGT GCT TAC CAC CAC GAG CAC CAC TTG CAC CCA ACT GTC CCA	840	TTC GGT GCT TAC CAC CAC GAG CAC CAC TTG CAC CCA ACT GTC CCA	850
AAG CCA CCA ATG GTG GTG CTC GTG GTG AAC GTC GGT TGA CAG GGT		P G G Y H H E H H L H P T V P>		P G G Y H H E H H L H P T V P>		P G G Y H H E H H L H P T V P>	
TGG TGG AGA TTG CCA TCC ACC AGA ACC AAG GGT GAC ACC GCT TAG	860	TGG TGG AGA TTG CCA TCC ACC AGA ACC AAG GGT GAC ACC GCT TAG	870	TGG TGG AGA TTG CCA TCC ACC AGA ACC AAG GGT GAC ACC GCT TAG	880	TGG TGG AGA TTG CCA TCC ACC AGA ACC AAG GGT GAC ACC GCT TAG	890
ATC ACC ACC TCT AAC GGT AGG TGG TCT TGG TTC CCA CTG TTG CGA ATC		W W R L P S T R T K G D T A *>		W W R L P S T R T K G D T A *>		W W R L P S T R T K G D T A *>	
TA AT							

FIGURE 16  
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FIGURES 17A  
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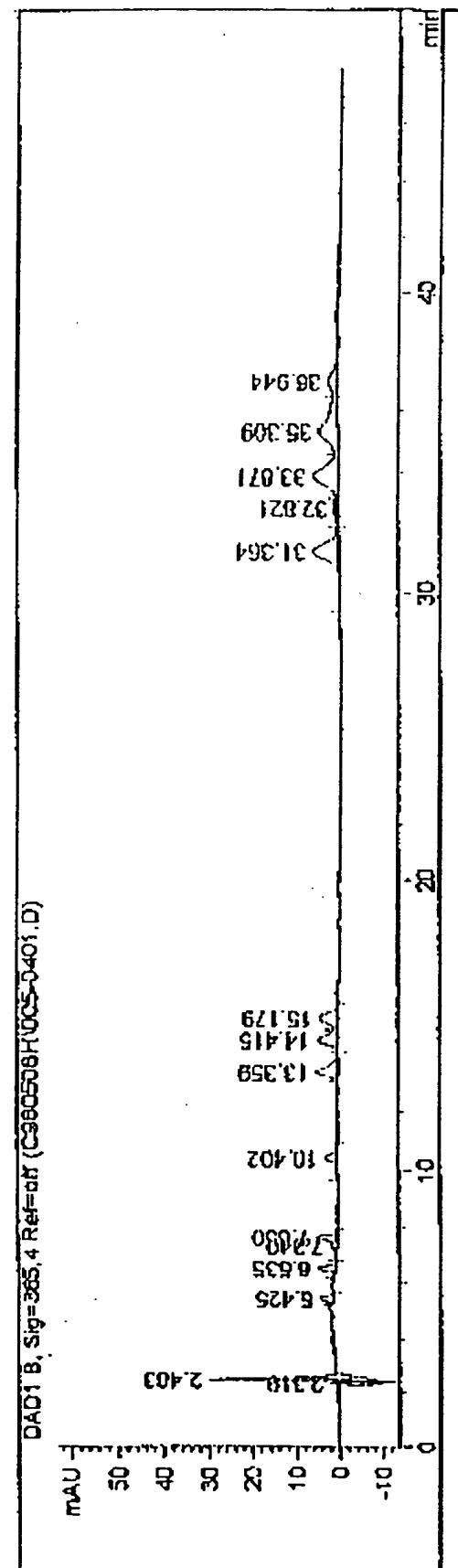


FIGURE 17B  
2/3

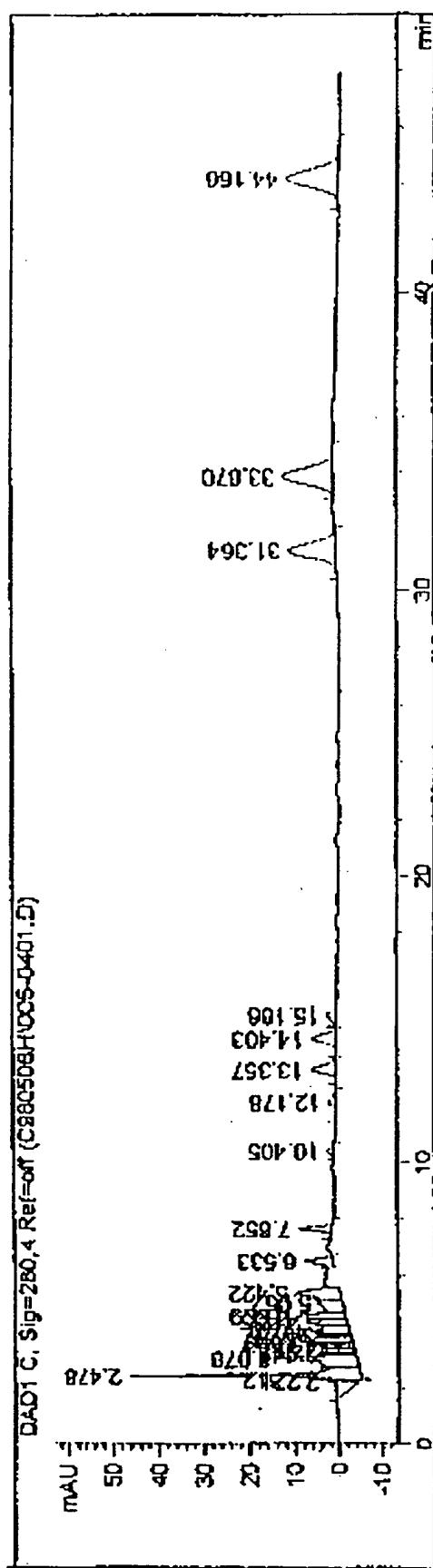


FIGURE 17C  
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## INTERNATIONAL SEARCH REPORT

Internat. Application No

PCT/US 98/16466

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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Y	WO 95 23863 A (CENTRE NAT RECH SCIENT ;CAMARA BILAL (FR); KUNTZ MARCEL (FR)) 8 September 1995 abstract, page 5, line 19-21; page 6,10,13 ---	18
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Intern. Appl. No.

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